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(54) Title: METHODS FOR ANALYSIS OF HUMAN STEM CELLS (57) Abstract The present invention provides methods for precisely and reproducibly assaying hematopoietic stem cell activity in a cell population and methods of using the assay. By expansion of hematopoietic stem cells identified by means of this assay, one may produce highly enriched or clonal populations of hematopoietic stem cells. The present invention also provides methods for producing enriched or clonal populations of other hematopoietic cell types, hematopoietic cells produced thereby, and methods of using the hematopoietic cells.		

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METHODS FOR ANALYSIS OF HUMAN STEM CELLSDESCRIPTIONTechnical Field

This invention is related to the analysis and
5 isolation of hematopoietic cells.

Background Art

Mammalian hematopoietic cells are responsible for an extraordinarily diverse range of activities. These cells are divided into several lineages, including lymphoid,
10 myeloid and erythroid. The lymphoid lineage, comprising B cells and T cells, produces antibodies, regulates cellular immunity, and detects foreign agents such as disease-causing organisms in the blood. The myeloid lineage, which includes monocytes, granulocytes, and
15 megakaryocytes, monitors the blood for foreign bodies, protects against neoplastic cells, scavenges foreign materials, and produces platelets. The erythroid lineage includes red blood cells, which carry oxygen.

Despite the diversity in the morphology, function,
20 and other characteristics of these cells, a single cell type, the hematopoietic "stem cell," is believed to act as the progenitor of all hematopoietic lineages. These rare primitive cells (approximately 0.01% of bone marrow cells) are distinguished by their high proliferative
25 potential and possible self renewal. Stem cells differentiate into multipotent progenitor cells and ultimately into each of the mature hematopoietic lineages. Thus, stem cells are believed to be capable of generating and sustaining long term hematopoiesis when
30 transplanted into immunocompromised hosts. Stem cells obtained from different sources such as adult bone marrow or mobilized peripheral blood may behave differently than similar fetal cells plated in single cell culture under

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identical conditions, it can be said that "stem cells" are those cells which exhibit the most primitive behavior observed.

The stem cell was originally defined by the capacity to self-renew and to give rise to progeny that are the committed precursors for all hematopoietic lineages. A number of researchers have concluded from their attempts to divide the progenitor cell compartment into stem cell and committed progenitor cells that these compartments constitute a hierarchy or continuum of cell types whose maturation is characterized by decreases in both pluripotentiality and the ability to repopulate the hematopoietic system of serially transplanted animals.

Strategies for isolating stem cells typically seek to exploit differences in cell size, density and the selection or depletion of cells based on the expression of cell surface antigens. It has been difficult, however, to identify and purify stem cells because of the small proportion of stem cells in the bone marrow, peripheral blood, and other sources. In addition, many cell surface antigen "markers" associated with stem cells are also present on more differentiated cells.

The marker, CD34, for example, is thought to be present on all human hematopoietic progenitor cells (Civin et al. (1984) J. Immunol. 133:157), and this "CD34⁺" population can mediate engraftment of an immunocompromised host in vivo (Berenson et al. (1991) Blood 77:1717-1722). CD34 is also present on endothelial cells. The fraction of the CD34⁺ compartment containing stem cells has not been consistently and reliably defined. Although the presence of primitive hematopoietic cells expressing relatively high CD34 density has been reported (Berenson et al. (1991); Terstappen et al. (1991) Blood 77:1218-1227; Teixido et al. (1992) J. Clin. Invest. 90:358-367), the CD34⁺ cell

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population is heterogeneous with respect to the types of progenitor cells and their relative state of differentiation. Terstappen et al. (1991).

Previously described schemes for obtaining stem cells require the sequential isolation of subpopulations of CD34⁺ cells which either have additional markers associated with stem cells or which lack markers associated with committed cells. Several schemes to fractionate human hematopoietic cells into lineage committed and non-committed progenitors have been reported. Berenson et al. (1991); Terstappen et al. (1991); Hoffman et al. (1988) J. Clin. Invest. 82:1017-1027; Landsdorp and Dragowska (1992) J. Exp. Med. 175:1501-1509; and Baum et al. (1992) Proc. Natl. Acad. Sci. USA 89:2804-2808. Unfortunately, such methods are technically complicated and often do not permit the recovery of enough stem cells to address multilineage differentiation along the different lymphoid and myeloid pathways. The sequential fractionation steps result in dramatic reductions in stem cell yield from a cell population which initially contains only about 0.01% of stem cells.

The relative paucity of stem cells in biological sources has prevented extensive research on stem cells and hematopoietic differentiation in general. The ready availability of a cell population enriched in stem cells would make possible the identification of biological modifiers affecting stem cell behavior. For example, there may be as yet undiscovered growth factors associated with (1) early steps of dedication of the stem cell to a particular lineage; (2) the prevention of such dedication; and (3) the ability to control stem cell proliferation.

The availability of sufficient numbers of stem cells in an enriched population would also be extremely useful,

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for example, in reconstituting hematopoiesis in patients undergoing treatments which destroy stem cells, such as cancer chemotherapy. Stem cells are also important targets for gene therapy.

- 5 Compounding the difficulty of providing sufficient numbers of stem cells for research and practical applications has been the lack of a precise and reliable means for assaying totipotent stem cells in vitro. There have been numerous attempts to develop assay systems.
- 10 Examples of in vivo assays include the measurement of the marrow repopulating ability (MRA) of cells or the ability to form colonies in the spleen (colony forming units-spleen or CFU-s) after transplantation into lethally irradiated mice. In another approach, in vitro colony
- 15 forming cell (CFC or CFU) assays measure the clonal growth of cells in semi-solid media (i.e., in agar or methylcellulose) or in association with pre-formed bone marrow stromal layers. However, these attempts have led to a great deal of confusion regarding the relationship
- 20 of progenitors to one another, a problem compounded by differences in read-out times, replating times, and analysis of the potentiality of cells produced in these assays. Even where hematopoietic reconstitution has been achieved, none of these studies have shown whether the
- 25 reconstituted hematopoietic lineages (in in vivo reconstitution assays) or colony or clonal formation (in vitro culture assays) are derived from a single totipotent stem cell or from long-lived lineage restricted progenitors.
- 30 Although a short-term repopulating donor cell population may suffice for some purposes, for others, e.g., gene therapy and research into stem cell regulation, the identification and isolation of the stem cell is essential. Moreover, the reliable identification
- 35 of stem cells would allow researchers to better identify,

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for example, cell surface antigens characteristic of stem cells or to clone genes whose expression is associated with stem cell self-renewal, commitment, and differentiation. Furthermore, in order to assure that one has truly obtained a stem cell, an effective stem cell assay is needed.

DISCLOSURE OF THE INVENTION

The present invention provides methods of assaying hematopoietic stem cell activity comprising the steps of plating a single input hematopoietic cell in an appropriate culture system, allowing the input cell to expand to produce progeny cells; analyzing the number and phenotype of the progeny cells and scoring the input cell as a stem cell if the input cell possesses the ability to proliferate from a single cell to approximately 8,000 progeny cells or more.

Additional criteria for scoring an input cell as a stem cell are: the potential of the input cell to produce both myeloid and lymphoid progeny cells; whether cells with a primitive immunophenotype are retained after expansion of the input cell; and whether cells with a primitive proliferative behavior are retained after expansion of the input cell.

Such assay methods may further comprise the steps of selecting a progeny cell, preferably by fluorescence activated cell sorting (FACS); replating the selected progeny cell in an appropriate culture system, whether by single cell plating or in bulk; and allowing the replated progeny cell to expand.

The present invention also provides an isolated stem cell identified by such an assay method and capable of producing at least 2×10^6 progeny cells.

Another embodiment of the invention is a method for evaluating a sample for the presence of a biological

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modifier affecting a biological response of a hematopoietic cell. The method comprises the steps of: plating a hematopoietic cell (e.g., a stem cell) produced by the method described above or a progeny thereof as a
5 test cell in an appropriate culture system along with the sample; plating a similar hematopoietic cell or a progeny thereof as a control cell in an appropriate culture system without said sample; and comparing the biological response of the test and control cells. In such a method
10 the test and control hematopoietic cells may be allowed to expand to produce progeny cells, in which case the comparison will likely comprise determining the number and phenotype of the progeny cells.

Clonal or enriched populations of hematopoietic
15 cells produced according to the methods of the present invention are useful, for example, in treating an animal affected by a genetic disease by a method comprising introducing into the animal such a hematopoietic cell or its progeny, wherein the stem cell or progeny thereof is
20 transfected with a nucleic acid capable of either expressing in said transfected cell a polypeptide which is missing or defective in said animal or a expressing a nucleic acid or polypeptide capable of inhibiting the expression of a target protein in said animal.

25 The present invention also provides a method of monitoring stem cell activity during the course of a medical treatment comprising determining the stem cell activity of a hematopoietic cell sample of a patient before the commencement of the treatment (to establish a
30 baseline) and during the course of the treatment (to obtain a test level) by an assay method of the present invention and comparing the baseline and test levels.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 outlines the visual (microscopic) analysis of hematopoietic proliferation after single cell plating.

Figures 2 and 3 provide phenotypic summaries of individual confluent wells, categorized by the number of days needed to reach confluence ("DAYS TO CONFL."). Also shown are the percentage of cells in each well which were CD34⁺/Lin⁻ ("%34+,LIN-") or CD19⁺/CD10⁺ B cells ("%19+10+") and, for wells with CD34⁺/Lin⁻ progeny (blast cells), the number of weeks for which blast cell maintenance in secondary culture may be demonstrated ("2NDARY PLT. BLAST CELL MAINT.").

Figure 4 shows FACS analyses of single CD34^{hi}/Lin⁻ cells from fetal bone marrow SF268 which expanded to confluence in individual wells at week five of culture. Plot #1: a portion of the cultured cells stained with the isotype control antibodies mouse IgG1-PE (Y axis) and IgG1-FITC (X axis). Plots #2 and #3: individual wells stained with anti-CD33-PE (Y axis) and anti-CD19-FITC and anti-CD10-FITC (X axis). Plots #4, #5, #6, and #7 are examples of individual wells stained with anti-CD34 plus goat anti-mouse IgG3-Texas red (Y axis) and anti-Lin-FITC (X-axis). Plot #4 shows the isotype control staining.

Figure 5 shows FACS analyses of a cell from the CD34^{hi}/Lin⁻ population from human fetal bone marrow SF293 sorted at one cell per well into a 72 well Terasaki plate. Figure 5A shows the population selected for plating (inside R2). CD34 staining is shown on the Y axis. The reanalysis of the sorted population is also shown. Figure 5B shows FACS analysis of one well (well #9) which reached confluence by week four of culture (CD34 staining is on the Y axis and Lin staining is on the X axis). The isotype control staining is shown for comparison. Figure 5C shows FACS analysis of two wells from the secondary plating which reached confluence at 4

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(panel 1) and 5 weeks (panel 2) after secondary plating, respectively. Isotype control stainings are shown in Figure 5C, panel 3.

Figure 6 shows secondary expansion data for a series of secondary cultures of the CD34⁺/Lin⁻ portions of expanded single cells reaching confluence at 0-17 days (Figure 6A), by 28 days (Figure 6B), between 39 and 45 days (Figure 6C), and after 42 days (Figure 6D).

Figure 7 is a flow diagram of the method used to determine the correlation between quiescence and potency of progenitor after in vitro expansion as described in Example 7.

Figure 8 shows gating strategy for sorting CD34⁺/Lin⁻ dye bright versus dye dim cell populations from PKH26 labeled CD34⁺/Thy⁺/Lin⁻ cells expanded in culture for 16 days:

- panels A-C Cadaveric DM7090A;
- panels D-F MPB 8814;
- panels A and C isotype control staining (no CD34 antibody);
- region 1 defines the CD34⁺/Lin⁻/dye dim population;
- region 2 defines the CD34⁺/Lin⁻/dye bright population.

Figure 9 shows detection of CD34⁺ cells after expansion of a single adult stem cell to ~ 8,000 cells:

- panels 1 and 2 show gating of the "blast" and "live" populations;
- panel 3 shows isotype control staining (no CD34 antibodies);
- panel 4 shows positive control (CD34 staining of KG1a);
- panel 5 shows a confluent adult well containing a CD34⁺ population;

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panel 6 shows a confluent adult well containing no detectable CD34⁺ population.

Figure 10 shows single adult stem cells can produce up to 1,000 CD34⁺ progeny:

- 5 panel 1 shows isotype control (no CD34 antibody);
panel 2 shows pooled progeny of 6 cells (contains 10% CD34⁺ cells);

calculation follows showing 1,000-fold expansion of CD34⁺ cells.

- 10 Figure 11 shows average plating efficiencies in single cell assay for dye bright (quiescent) and dye dim (proliferative) CD34⁺/Lin⁻ ABM and MPB cells after in vitro expansion.

- 15 Figure 12 (numbers for graphs were obtained by method in the last paragraph of Example 3) shows average cell production in single cell assay from CD34⁺/Lin⁻/dye bright cells versus CD34⁺/Lin⁻/dye dim cells.

Figure 13 shows primitive colony forming ability of expanded progeny dye bright cells.

20 MODES FOR CARRYING OUT THE INVENTION

Assay System

- An assay system has been developed which provides a significant improvement over available technology for determining the proportion of stem cells or other
25 hematopoietic progenitors in a mixed cell population.

- In this assay system, input (test) cells are plated at one cell per well in an appropriate culture system. The culture system can be any combination of stroma, growth factor, surface, and basal medium that allows
30 input cells to exhibit the measured behavior (e.g. growth or lineage commitment), preferably a AC6 stromal co-culture (see Baum et al., "Long-Term In Vitro Lymphocyte

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Cultures," copending patent application U.S.S.N. 07/938,548, filed 8/28/92), preferably containing cytokines including, but not limited to, interleukin 3 (IL-3), interleukin 6 (IL-6), steel locus factor (SLF), and leukemia inhibitory factor (LIF). Surface refers to various surfaces or surface treatments including but not limited to attachment factors and plastic surfaces with various charges. The culture may also contain additional factors including, but not limited to, erythropoietin, colony stimulating factors, interleukins, γ -interferon, or antibodies against transforming growth factor, or include other cells which secrete factors involved in such biological responses as stem cell regeneration, commitment, and differentiation, or medium conditioned by such cells.

The time course and phenotypic outcome of each well is analyzed. The time course of growth, i.e., cell number and morphology, is observed at regular intervals, i.e., every two days or more frequently, by visual examination, which may be aided by an image acquisition and analysis system. Visual examination can distinguish, for example, cell size, morphology, and the ability to move within a plate. Cell types can be further identified by the observed degree and time course of proliferation. The number of total cells or subcategories of discernible cell types is determined.

After some expansion, cells can be stained for cell surface markers, followed by microscopic fluorescent measurement or FACS analysis. In-well staining is preferred for fluorescent microscopy, although cells may also be transferred to a secondary slide or plate. The combination of data from proliferation kinetics and staining of the progeny unambiguously identifies the input cell, e.g., as a primitive progenitor cell or a more differentiated cell type. While it is possible to

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analyze expanded cells at an arbitrary time point (e.g., 5 weeks after plating), it is preferable to analyze cells based on the extent of proliferation, e.g., reaching confluence in a Terasaki well. When dye-labeled single fetal or adult stem cells are used, asymmetric division was observed during expansion.

In addition, the progeny cells can be studied by, e.g., micromanipulator harvest of cells of interest or replating at a single cell per well to further define the proliferative potential of the input cell.

The resulting data indicate the proliferative potential and ability to form multiple lineages (e.g., B cells and myeloid cells), as well as detecting quiescent behavior, thus defining in vitro stem cell behavior.

Extremely precise data on the proliferative potential and range of candidate stem cells can be obtained by plating one cell per well, expanding the plated cells, individually resorting wells derived from the various expanded cells, and replating at one cell per well, and repeating this cycle as long as cells with high proliferative activity are retained after expansion. This is a significant advance over methods in common use that utilize bulk expansion followed by replating, resorting, and/or phenotypic analysis for several reasons: (1) bulk culture methods do not clearly detect quiescence and thus fail to clearly distinguish between the persistence of quiescent stem cells and the presence of other cells that have high or long-lived proliferative potential; (2) bulk culture methods do not clearly establish that multiple lineages arise from a single cell; and (3) bulk culture methods provide no information regarding the maximum proliferative potential or the range of potentials of individual cells within a mixture of cells. The method described above has clearly demonstrated the ability of a single fetal stem cell to

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generate as many as 2×10^7 cells in vitro, a proliferation approximately 10- to 100-fold greater than what has been previously obtained. The assay methods of the present invention therefore clearly indicate the

5 proliferative potential of putative stem cells with far greater precision, better distinguishing stem cells from other long lived progenitor cells and other cells having proliferative potential. The methods of the present invention also enable the rigorous detection of in vitro

10 stem cell self-renewal, defined as the production of multiple stem cells with proliferative range and potential equal to that of a single stem cell input cell. Self-renewal is demonstrated by the ability to expand in culture and resort for stem cells through multiple

15 cycles, with each cycle generating an increase in the number of cells that display stem cell behavior.

Time lapse image analysis has allowed a correlation of the early patterns of growth from single cells with later verification of stem cell potential, such as

20 expansion capacity, production of both B lymphoid and myeloid cells, and replatability. The most primitive fetal cells display an early dispersed growth pattern. Adult cells exhibit the same phenomena, except that the number of cells produced before motility and dispersal

25 ceases and the formation of the cobblestone areas begins lower (10-200 cells for adult vs. 50-800 cells for fetal). Therefore, the number of cells produced before motility ceases may prove to be an early indicator of potency.

30 Adult $CD34^+/Lin^-$ cells which show quiescent behavior (remain dye bright after a 16 day culture period) exhibited a higher plating efficiency when subsequently plated in the single cell assay than proliferating (dye dim) $CD34^+/Lin^-$ cells. The average plating efficiency from

35 four tissues was 53% for dye bright cells and 27% for the

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dye dim cells from stroma free bulk cultures as well as cells from AC6.21 cocultures (Fig. 11).

The average cell production from 72 dye bright cells cultured in the single cell assay for four weeks was
5 1,800, while the average cell production from dye dim cells was 320 (Fig. 12). Similar behaviors were observed after two 16-day cycles of bulk expansion, indicating that quiescent cells with high proliferative potential can exist in bulk culture for at least four weeks.
10 Expanded populations from single dye bright cells retained primitive colony forming activity (Fig. 13).

This novel method for assessing potency of cells in hematopoietic cell mixtures after expansion, may be useful for predicting engraftment potential in products
15 intended for cellular therapy.

The assay methods of the present invention allow one to score a cell as a stem cell by the following criteria: (1) the cell proliferates from a single cell to approximately 20,000 cells or more (fetal), or 8,000
20 (adult); (2) the cell produces both myeloid and lymphoid progeny; (3) cells with a primitive immunophenotype are retained, and (4) cells with a primitive proliferative behavior are also retained (e.g., single cells re-isolated after primary expansion are able to further
25 expand upon replating).

Using the described assay, fetal stem cells meeting criteria (1) to (4) have often been identified. In this assay, adult stem cells commonly meet criteria (1) and (2) and less commonly (3). Cells, particularly fetal
30 cells, meeting all four criteria are scored as stem cells. Adult cells displaying fewer defining behaviors, at least (1) and, preferably, (2) are also scored as stem cells. The differences in the observed proliferative capability of fetal and adult stem cells may reflect
35 inherent limitations in adult stem cells, a different

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need in response to growth or attachment factors, or other unknown variables. In any case, the claimed assay methods (using any applicable culture conditions) can be used to determine the maximum proliferative potential and
5 range of proliferative potential for cells in a given hematopoietic population, and thus can be used to determine stem cell behavior in populations that are known to include stem cells.

For adult cells, the primitive behavior is lower
10 than that for the fetal cell:

(1) in at least 15 experiments with different tissues, testing some 5,000 individual adult stem cells, no one cell has been observed to expand to the extent of forming multiple cell layers in the Terasaki well
15 (>10,000 blast cells). Instead the most highly proliferating cells generally regressed just before reaching confluence (approximately 8,000 cells). See Fig. 8, showing blast cell expansion from one cell to 8,000.

20 (2) As with expansions from single fetal cells, myeloid cells (CD33⁺) were found in 100% of expansions from single adult cells. However, detectable B lymphoid populations (CD19⁺) were rarely found in confluent adult wells. In contrast 15% of confluent fetal wells
25 contained a detectable CD19⁺ population. It is possible that adult CD19⁺ cells are lost before confluence is reached, as cells with lymphoid morphology have been observed in some sparsely populated wells which later apoptosed.

30 (3) Approximately one third of confluent wells from fetal serum expansions contained a detectable expanded CD34⁺/Lin⁻ population, while detection of CD34⁺ cells in adult confluent wells was very rare (< 1% of wells analyzed). Fig. 9 shows a FACS analysis of a well that

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contained a detectable CD34⁺ population (well 1) and one that did not (well 2). Preliminary evidence from in situ staining of cells in Terasaki wells suggests that most adult CD34⁺ cells produced during expansion differentiate
5 before the well reaches confluence, thus explaining the low frequencies of CD34⁺ cells in confluent wells. In adult expansions where CD34⁺ cells were detected, up to a 1000 fold expansion of CD34⁺ cells from a single adult stem cell had been observed (Fig. 10 shows calculation).
10 For fetal stem cells this number has been as high as 8,000 fold.

(4) Due to the low frequency of confluent wells containing detectable CD34⁺ populations, secondary plating has not been possible with adult cells.

15 Therefore, the most primitive behavior observed with adult cells was the ability to produce at least 8,000 cells with a blast cell morphology from a single cell using the culture method described. Approximately 1-3% of adult bone marrow CD34⁺/Thy⁺/Lin⁻ or CD34⁺/CD38⁻ cells
20 met this criteria.

By means of the claimed assay methods a stem cell can be precisely and reliably identified and expanded. The enablement of research into the phenotype (e.g., possession of cell surface antigens identified by
25 immunological means) and behavior of such stem cells is itself of great importance.

The availability of stem cells or enriched or clonal populations of other hematopoietic cells made possible by the present invention and a more complete understanding
30 of their biology, greatly assists in the development of improved assays for stem cell activity; optimization of hematopoietic cell processing strategies; and identification of culture conditions and biological modifiers (e.g., polypeptides, peptides, nucleic acids,

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or small molecules) which affect such biological responses as the proliferation, commitment, differentiation and maturation of stem cells. Such an advance also makes possible, for example, the

5 identification of markers or other target antigens present on, and preferably specific for, a given hematopoietic cell type; and the identification and cloning of genes whose expression is associated with biological responses of hematopoietic cells, including

10 the proliferation, commitment, differentiation and maturation of stem cells.

PKH26 Dye Labeling

PKH26 has not heretofore been used to document asymmetry of division of stem cells by imaging, or to

15 compare the proliferative potential of cells from the same tissue with the same immunophenotype but a different division history in culture. See for example, Landsdorp et al. (1993) J. Exp. Med. 178:787; and Landsdorp et al. (1993) Exp. Hemat. 21:1321.

20 Production of Highly Enriched Hematopoietic Cell Populations

The present invention also permits the production of highly enriched or clonal hematopoietic populations.

A cell population enriched in stem cells, e.g.,

25 resulting from FACS sorting of human fetal bone marrow, is plated at one cell per well in an appropriate culture system, preferably in Terasaki well cultures in the presence of AC6 stroma and IL-3, IL-6, SLF, and LIF. Additional factors may be added, including but not

30 limited to IL-1, IL-7, IL-11, G-CSF, GM-CSF and an IL-3/GM-CSF fusion protein. Single cells from wells in which the single original input cell was a stem cell, as determined by the assay described above, may be used

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directly, preferably after being resorted, or resorted and replated either as single cells or in bulk in a secondary culture under appropriate conditions and expanded to produce large numbers of progeny. These
5 expanded cell populations are highly enriched in stem cells. By such means one may readily obtain a clonal population, or at least a highly enriched population, of stem cells.

Further, subsequent bulk sort-and-expand cycles may
10 be conducted in increasingly larger culture vessels as long as stem cell self-renewal occurs. The bulk separation steps may include FACS or other cell separation technology to isolate the desired phenotype for each expansion cycle; a variety of culture methods
15 are available to accomplish bulk stem cell expansion.

Alternatively, this technology may be used in order to obtain a pure (i.e., clonal) or highly enriched population of another hematopoietic cell type, e.g., lymphoid or myeloid cells. For such purposes, one
20 identifies a combination of input cell, growth conditions, and reisolation conditions which, after reisolation, gives rise to a highly enriched or clonal population of the desired hematopoietic cell type. As discussed in Example 5 below, the data from secondary
25 replating show dramatic enrichment of certain cell types and behaviors, e.g., blast-morphology macrophage progenitors with a consistent time period to generation of macrophages in culture.

Preparation and propagation of cell populations enriched
30 in stem cells

The claimed stem cell assay methods preferably begin with cell populations enriched in hematopoietic stem cells, although for some applications one may apply the assay methods to non-enriched cell populations, such as

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bone marrow or blood cells. These enriched populations can be prepared from a variety of adult, fetal, or neonatal hematopoietic cell sources, including but not limited to bone marrow, fetal liver, embryonic yolk sac, fetal and adult spleen, and blood, using any applicable cell purification methods. Bone marrow cells may be obtained from the tibia, femur, spine, or other bone cavities.

The enrichment process may employ any of the strategies well known in the art. Phenotypes that have been reported in the literature to define the most primitive hematopoietic cells include CD34⁺/Thy-1⁺, CD34⁺/CD38^b, and CD34⁺/CD45RA⁻. CD34⁺ cells will preferably have a high density of CD34, preferably 100-fold or more over levels on isotype controls. CD34^{hi} cells are described in DiGiusto et al. "Method for Producing a Highly Enriched Population of Hematopoietic Stem Cells and Stem Cell Populations Derived From. Stem cells are also Lin⁻. "Lin⁻" refers to the absence or low expression of markers associated with lineage committed cells, including, but not limited to, T cells (markers such as CD2, CD3 or CD8); B cells (markers such as CD10, 19 or 20); myelomonocytic cells (markers such as CD14, 15, 16); natural killer ("NK") cells (such as CD2) and red blood cells ("RBC") (such as glycophorin⁺) megakaryocytes, mast cells, eosinophils and basophils. CD34^{hi} cells from fetal tissue have low levels of CD38; low or intermediate levels of CD13; and no appreciable CD14, 15, 16, or glycophorin A markers.

Table 1 summarizes probable phenotypes of stem cells in fetal, adult, and mobilized peripheral blood. In Table 1 myelomonocytic stands for myelomonocytic associated markers, NK stands for natural killer cells and AMPB stands for adult mobilized peripheral blood. As

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used herein both infra, supra and in Table 1, the negative sign or, uppercase negative sign, (-) means that the level of the specified marker is undetectable above Ig isotype controls by FACS analysis, and includes cells
5 with very low expression of the specified marker.

Table 1

Table 1																	
Probable Stem Cell Phenotypes																	
NK and T cell markers						B cell markers			Myelomonocytic				Other				P-gp Activity
CD2	CD3	CD8		CD10	CD19	CD20	CD14	CD15	CD16	CD33	CD34	CD38	HLA-DR	C-Kit	Thy	Rho	
-	-	-		-	-	-	-	-	-	?	+	-	+	+	+	+	+
FBM																	
ABM	-	-		-	-	-	-	-	-	-	+	?	lo/-	+	+	lo	+
AMPB	-	-		-	-	-	-	-	-	lo/-?	+	?	lo/-	?	+	lo	+

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Hematopoietic cells are preferably fractionated into various subpopulations by FACS, especially FACS employing a limited panel of antibodies to highly autofluorescent myeloid cells to increase sorting resolution. See, e.g.,

5 Flow Cytometry and Sorting, ed. Melamed, Lindmo, and Mendelsohn, Wiley-Liss, Inc., 1990, especially the articles by Lindmo et al., pp. 145-169, and Visser, pp. 669-683. A single step selection for CD34^{hi} cells by flow cytometry, for example, will generally achieve a cell

10 preparation having at least about 0.5% stem cells.

Preferably, FACS, more preferably multi-color analysis using FACS, is employed to identify and/or select cells having the desired immunophenotype or other physical characteristics. The antibody for one marker,

15 e.g., CD34, is typically labeled with one fluorochrome, while antibodies specific for the various dedicated lineages, if used, are conjugated to one or more different fluorochromes. Fluorochromes which may find use in a multi-color analysis include phycobiliproteins,

20 including but not limited to phycoerythrin and allophycocyanins, fluorescein and Texas red.

The selected cells may be further fractionated to achieve even more highly purified stem cell populations by one or more additional selections for markers (or

25 other characteristics) associated with the desired cell type, e.g., stem cells, or against other markers, e.g., those associated with lineage committed or mature hematopoietic cells. Additional selections may be performed sequentially for several markers or may be

30 selected for (or against) in a single step.

Although desired hematopoietic cell types may be separated from other cell types by cell cytometry, a preliminary separation may be employed to remove lineage committed cells (e.g., T cells, pre-B cells, B cells, and

35 myelomonocytic cells, or minor cell populations, such as

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megakaryocytes, mast cells, eosinophils and basophils) and enrich the cell population for the desired cell type before directly selecting for stem cells. Typically, platelets and erythrocytes are removed prior to sorting.

- 5 It is not essential to remove every other cell class, particularly minor cell populations, although preferably at least about 70% and preferably at least 80% of the cell types that are not desired will be removed. Preliminary separations may conveniently be performed, 10 for example, using magnetic beads coated with one or more specific monoclonal antibodies.

- Dead cells may be selected against by employing such dyes as propidium iodide (PI). Stem cells have low side scatter and low forward scatter profiles as determined by 15 FACS analysis. Cytospin preparations show that stem cells have a size between mature lymphoid cells and mature granulocytes. Cells may be selected based on light-scatter properties as well as by their expression of various cell surface antigens.

- 20 Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies may be attached to a solid support to facilitate preliminary separation. The separation 25 techniques employed should maximize the retention of viability of the fraction to be collected.

- Antibodies employed for cell separations may be labeled by any method known in the art. Useful labels include but are not limited to fluorochromes, biotin, or 30 other widely used labels. Alternatively, antibodies may be affixed to a solid support such as magnetic beads, which allow for direct separation.

- The particular preliminary separation technique employed will depend upon efficiency, ease and speed of 35 performance, and the need for sophisticated equipment

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and/or technical skill. The techniques useful for preliminary separations include but are not limited to magnetic separation using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a
5 monoclonal antibody or used in conjunction with a monoclonal antibody, e.g., complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., plate, or other convenient technique. Separations can also be effected by exploiting differences in physical
10 properties including but not limited to density gradient centrifugation and counter-flow centrifugal elutriation and vital staining properties including but not limited to rhodamine-123 and Hoechst 33342. Techniques providing more accurate separation include FACS, which can have
15 varying degrees of sophistication, including but not limited to a plurality of color channels, low angle and orthogonal light scattering detecting channels, impedance channels, etc.

After a desired hematopoietic cell type,
20 particularly a stem cell, is obtained by the claimed methods, one may wish to employ subsequent bulk sort-and-expand cycles in order to create increasingly larger populations of the desired cell type, e.g., in bioreactors. For each cycle, bulk separation methods are
25 used to isolate the cell type of interest, including FACS, column-based immunophenotype selection, magnetic separation technologies, or methods based on physical parameters such as size or density.

For isolation of bone marrow from fetal bone or
30 other bone source, the bone may be flushed with an appropriate balanced salt solution, preferably supplemented with fetal bovine serum (FBS) or other source of naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally
35 from about 5-25 mM. Convenient buffers include but are

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not limited to Hepes, phosphate buffers, and lactate buffers. Otherwise bone marrow may be aspirated from the bone in accordance with conventional methods.

When antibodies are used for positive or negative
5 selection of cells from bone marrow, the bone marrow cells are typically incubated for a short period of time at reduced temperatures, generally about 4°C, with saturating levels of antibodies specific for markers. The cells are then washed with a FBS cushion, suspended
10 in an appropriate buffered medium, then separated by means which recognize bound antibodies specific for particular cell surface antigens.

Isolated stem cells may be propagated in a medium containing maintenance factors supporting the
15 proliferation of stem cells, such as the growth factors secreted by stromal cells, which can be obtained from bone marrow, fetal thymus or fetal liver and which can be allogeneic or xenogeneic. For that reason, isolated stem cells may be propagated by growth in media conditioned by
20 stromal cells or by co-culturing with stromal cells. Stromal cells used in such co-cultures may be clonal cell lines (e.g., AC3 or AC6, also known as SyS-1 or AC6.21) or mixed stromal cell preparations derived from mouse or human fetal bone marrow from which hematopoietic cells
25 have been removed. For example, hematopoietic cells can be removed by employing appropriate monoclonal antibodies conjugated with toxin, antibody and complement, etc., and then selected for the ability to maintain human stem cells.

30 Hematopoietic cells, e.g., stem cells, may be frozen in liquid nitrogen and stored for long periods of time in 10% DMSO, 50% FBS, 40% RPMI 1640 medium. Once thawed, the cells may be grown in an appropriate culture system.

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Uses for the Claimed Assay Methods and Enriched Hematopoietic Cell Preparations

Among the uses of the methods and compositions of the present invention are the following.

- 5 Drug discovery. The stem cell assays of the present invention may be adapted in order to identify culture conditions or biological modifiers such as growth factors which promote or inhibit such biological responses as self-regeneration, proliferation, commitment, differentiation, and maturation of stem cells or other hematopoietic cells. In this way one may also identify, for example, receptors for these biological modifiers, agents which interfere with the interaction of a biological modifier and its receptor, and factors or antisense polynucleotides affecting gene transcription or translation. One may also use such assays as a strategy to identify and clone genes the expression of which affects the self-regeneration, proliferation, commitment, differentiation, and maturation of stem cells or other hematopoietic cells.

- To identify a biological modifier in a test sample, a hematopoietic cell type, e.g., a stem cell, identified by the claimed assay methods or progeny thereof, is plated as a single cell in the culture system of the present invention along with the test sample and allowed to expand to produce progeny cells. The rate of activation, proliferation, differentiation, and maturation (or other pertinent biological responses) of the hematopoietic cell(s) is compared to that of hematopoietic cell(s) cultured under control conditions, e.g., by determining the number and phenotype of progeny cells by flow cytometry.

- It is important to emphasize that the assay methods disclosed herein are useful not only to determine stem cell activity but, more generally, to assess the entire

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range of cell types and proliferative potentials that exist in a sample of hematopoietic cells (including pre-procedure and post-procedure clinical samples). The alternative is generally to perform all of the applicable
5 clonogenic assays, such as CFU-G, in limiting dilution or a similar bulk format.

One may also assay for cell-cell interactions, whether such interactions depend on contact between one cell type and another or simply the production of one or
10 more biological modifiers by a cell type into the medium shared by another cell type. To assess such cell-cell interactions, a first hematopoietic cell type is plated together with a second cell type, and it is determined whether an activity of the first cell type is affected by
15 the presence of the second cell type. For example, one could use such an assay to discover a stromal cell type or a stromal cell substitute which promotes self-renewal of stem cells or some other proliferative outcome.

In assays for hematopoietic stem cell activity and
20 for biological modifiers affecting a biological response of a stem cell, one commonly allows the stem cells to expand and then examine, for example, the number and immunophenotype of the progeny cells. This is often followed by the reisolation of a target cell population,
25 which may, itself, be allowed to expand. However, the primary expansion to confluence and reisolation of a target cell population are unnecessary for a number of useful assays. Such steps may be unnecessary, for example, in assays for the activity of hematopoietic
30 progenitors other than stem cells or for assays of growth factors that act on hematopoietic cell types other than stem cells.

The capacity of stem cells to differentiate into various hematopoietic lineages may be demonstrated by
35 culturing cells identified as stem cells using the assay

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system described above and in the Examples under appropriate conditions.

For example, in order to measure the effect of erythropoietin (EPO) on erythroid progenitors (BFU-E) isolated by FACS for other techniques, one may plate the isolated cells at a single cell per well into test (+ EPO) versus control (- EPO) conditions, then, over time, observe the proliferation and cell morphology of the cells and, if necessary, analyze the immunophenotype of progeny cells to fully determine proliferative outcome. This latter analysis may be unnecessary for a given assay if sufficient data are available, e.g., if the appearance of red blood cells caused by EPO is visually obvious.

Stem cells are typically grown on mouse or human stromal cells, although stroma-free culture systems are available. The Examples describe culture conditions that allow the expansion and terminal differentiation of stem cells into lymphoid and myeloid cells as determined by FACS, morphology, or staining combined with microscopy. The medium employed for the culturing of stem cells for these purposes can be any medium known in the art and is preferably a defined enriched medium, such as IMDM (Iscove's Modified Dulbecco's Medium) or a 50:50 mixture of IMDM and RPMI (a commonly used medium whose name refers to "Roswell Park Memorial Institute"), and is generally composed of salts, dextrose, amino acids, vitamins, 5×10^{-5} M 2-mercaptoethanol (2-ME), streptomycin/penicillin at 100 μ g/ml and 100 U/ml, respectively, and 10% FBS. The medium is typically changed from time to time, generally at least about once or twice per week.

The capacity of stem cells to differentiate into myeloid cells may be determined as set forth in the Examples below. To demonstrate the capacity of stem cells to differentiate into T cells, isolated fetal

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thymus fragments are cultured for 4 to 7 days at about 25°C in order to substantially deplete the thymus of its lymphoid population. Stem cells having human leukocyte antigen (HLA) mismatched with the HLA of the thymus cells are microinjected into the thymus tissue, which is then transplanted into a scid/scid mouse preferably under the Kidney capsule as described in EPA 0 322 240. The presence of mature T cells is best determined by FACS analysis of excised tissue for the presence of donor-derived CD3⁺, CD4⁺, and CD8⁺ cells.

The capacity of stem cells to differentiate into erythroid cells may be determined by conventional techniques to identify burst forming units-erythroid (BFU-E) activity, for example, methylcellulose culture. Metcalf (1977) In Recent Results in Cancer Research 61, Springer-Verlag, Berlin, pp. 1-227.

These assays may be readily adapted in order to identify substances such as growth factors which, for example, promote or inhibit stem cell self-regeneration, commitment, or differentiation.

Identification of target antigens associated with a specific hematopoietic cell type. The assay methods of the present invention permit the precise, unambiguous and reliable identification of various hematopoietic cell types, and highly enriched or clonal populations of these cell types are made available by clonal expansion of single cells so identified, followed, if necessary, by resorting of cells so identified. These advances permit the efficient evaluation of markers that distinguish individual hematopoietic cell types, particularly stem cells, from other cell types.

This is accomplished, for example, by using the cell as an antigen for raising antibodies, preferably monoclonal antibodies, by methods known in the art. These antibodies are then screened to identify those

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hybridomas producing monoclonal antibodies which distinguish that cell from other cells. In one approach candidate antibodies are evaluated by purifying the candidate antibody and conjugating it to a fluorophore, and using the antibody to test cells sorted by FACS for stem cell and progenitor content.

Such monoclonal antibodies would themselves be useful for a variety of indications. These include, but are not limited to, improved assays, for selecting for or against cells expressing their target antigen; for purifying the target antigen itself; and for gene cloning strategies, as discussed more fully below.

For techniques for the production and uses of antibodies see, e.g., Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH Laboratories or Goding (1986) Monoclonal Antibodies: Principles and Practice, 2d ed, Academic Press, New York. In vitro exposure of lymphocytes to the cellular antigen or selection of libraries of antibodies in phage or similar vectors may also be used. See, e.g., Huse et al. (1989) Science 246:1275-1281. Various methods of protein purification, including the uses of antibodies for immunopurification, are described in Guide to Protein Purification, ed. M. Deutscher, vol. 182 of Methods in Enzymology (Academic Press, Inc.: San Diego, 1990); and R. Scopes, Protein Purification: Principles and Practice, (Springer-Verlag: New York, 1982).

Gene cloning strategies. The cells of the present invention may be used to identify and clone genes whose expression is associated with proliferation, commitment, differentiation, maturation, or other characteristic biological responses of stem cells or other hematopoietic cells. Such cloning of recombinant genes may be done by any method known in the art including but not limited to subtractive hybridization, and expression cloning. Such

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recombinant genes would themselves be important reagents when used as probes to mark, e.g., stem cell activation, receptor expression, or the sequence of marker appearance and disappearance. For example, the assay systems of the present invention are used to identify quiescent stem cells, which are harvested using a micromanipulator. The patterns of expression of RNA and proteins in the harvested cells are then compared to that of a control cell, such as an activated (i.e., dividing) stem cell, to identify one or more differences in these patterns of expression.

Techniques for nucleic acid manipulation useful for the practice of the present invention are described in a variety of references including but not limited to in Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press (1989); and Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience: New York (1987) and periodic updates. Reconstituting hematopoietic cells or providing clonal cells of hematopoietic lineages. The availability of a highly enriched population of stem cells is also useful for reconstituting the full range of hematopoietic cells in an immunocompromised host following therapy including, but not limited to, radiation treatment or chemotherapy. These therapies destroy hematopoietic cells intentionally or as a side-effect of bone marrow transplantation or the treatment of lymphomas, leukemias and other neoplastic conditions.

The present invention also provides a source of highly enriched or clonal cells of specific hematopoietic lineages. The maturation, proliferation and differentiation of stem cells or other, "later" precursor cells into one or more selected lineages may be effected through culturing the cells under appropriate culture

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conditions, e.g., by the addition of appropriate factors, such as EPO, colony stimulating factors, e.g., GM-CSF, G-CSF, or M-CSF, SCF, interleukins, e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-11, IL-12, IL-13, an IL-3/GM-CSF fusion protein, γ -interferon, and antibody against transforming growth factor (anti-TGF β), and/or by co-culturing with stromal cells or other cells which secrete factors responsible involved in such biological responses as stem cell regeneration, commitment, and differentiation, or with cultured medium, e.g., recovered stroma culture.

Monitoring medical treatment. The assay methods of the present invention are also useful in monitoring stem cell activity in a patient undergoing medical treatment, such as radiation treatment or chemotherapy. Samples of hematopoietic cells, e.g., from the patient's bone marrow or blood are assayed for the presence of stem cells during the course of the treatment. The results of such an assay are preferably compared to a similar assay performed before the commencement of the medical treatment.

Gene therapy. Stem cells are also important targets for gene therapy. Expression vectors may be introduced into and expressed in autologous or allogeneic stem cells, or the genome of stem cells may be modified by homologous or non-homologous recombination by methods known in the art. The assay is also useful in documenting gene incorporation into stem cells.

Gene therapy may be used to correct genetic defects in an individual or provide genetic capabilities naturally lacking in stem cells. For example, diseases including, but not limited to, β -thalassemia, sickle cell anemia, adenosine deaminase deficiency and recombinase deficiency and recombinase regulatory gene deficiency may be corrected or treated in this fashion. Diseases not

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associated with hematopoietic cells may also be treated, diseases related to the lack of secreted proteins such as hormones, enzymes, growth factors, etc. Inducible expression of a gene of interest under the control of an appropriate regulatory initiation region will allow production (and secretion) of the protein in a fashion similar to that in the cell which normally produces the protein in nature.

Similarly, one may inhibit the expression or activity of particular gene products by methods known in the art including but not limited to, drugs and antisense oligonucleotide therapy. Drug resistance genes, including but not limited to the multiple drug resistance (MDR) gene, may also be introduced into stem cells, e.g., to enable them to better survive drug therapy of the patient into whom they have been reintroduced. For hematotropic pathogens, such as HIV or HTLV-I and HTLV II, the engineered stem cells can be genetically modified to prevent the proliferation of a pathogen in stem cells or differentiated cells arising from stem cells.

Alternatively, one may wish to remove or modulate expression of a particular DNA sequence from the genome of a stem cell, including but not limited to a variable region of a T-cell receptor from the T-cell repertoire by employing homologous recombination. Expression may be modulated by various methods known in the art including but not limited to DNA manipulation such as directed insertions, deletions and substitutions or by indirect effects such as by anti-sense technology.

The following examples are provided to illustrate but not limit the invention.

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Example 1: Isolation and Debulking of Human Bone Marrow or Peripheral Blood Cells and Fetal Bone Marrow

Human fetal tissues (18-23 weeks gestation) were obtained with informed consent from two agencies, the
5 International Institute for the Advancement of Medicine (Exton, PA) and Advanced Bioscience Resource (Alameda, CA) in compliance with state and federal regulations. The tissues were derived from curettage operation involving physical extraction without administration of
10 prostaglandins or related drugs. The tissues were individually placed in sterile 50 mL tubes containing processing medium (RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts), 50 U/mL penicillin, and 50 µg/mL streptomycin) at 4°C. The
15 samples were shipped on wet ice and received within 16-20 hours.

Bone marrow was removed from long bones within 4 hr. Briefly, bones were cut lengthwise and cells were flushed from the marrow cavity with a stream of ice cold
20 processing medium. Extracted cells were separated on a Ficoll (Pharmacia LKB, Uppsala, Sweden) density gradient (10^8 nucleated cells in 25 mL Hanks BSS (JRH Biosciences) with 2% FBS layered over 25 mL Ficoll). The mononuclear layer (density < 1.077) was extracted from the gradient,
25 washed once in 50 mL staining medium (RPMI 1640 without phenol red, 2% FBS, and 10 mM HEPES), then depleted of CD15-expressing cells using Dynal M 450 magnetic beads coated with anti-CD15 (antibody PM81, Mederex). Briefly,
30 10^8 mononuclear cells were mixed with 4×10^8 beads in 2 mL staining medium and incubated on ice for 30 min. with occasional mixing. The cell/bead mixture was then diluted to 10 mL staining medium and placed against a magnet (Advanced Magnetics Inc.) for one minute. The supernatant containing the unbound cells was removed to a
35 new tube and re-exposed to the magnet. The magnetic

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beads were washed once in 5 mL staining medium and the supernatants from the remagnetized sample and the wash were combined to obtain the "CD15 depleted population". The cells were then resuspended at a concentration of 2×10^7 /mL in staining medium.

Mobilized peripheral blood:

Apheresed mobilized peripheral blood samples were obtained with informed consent from patients treated at the University of Arkansas Medical Center. Multiple myeloma patients were treated on day 0 with cyclophosphamide (Cytosan[™]) at 6 g/m^2 (1.5 g/m^2 every 3 hrs x 4 doses). From day 1 until the start of leukapheresis (usually 10-28 days) granulocyte macrophage colony stimulating factor (GM-CSF) was given at 0.25 mg/m^2 /day. Apheresis for total white cells was started when the peripheral blood white cell count was greater than $500 \text{ cells}/\mu\text{L}$, and the platelet count was greater than $500,000/\mu\text{L}$.

About 3×10^8 cells were obtained from a small leukapheresis sample. Erythrocytes were lysed by incubation for 5 minutes with 0.83% ammonium chloride solution. A low density mononuclear cell fraction was prepared by using ficoll-hypaque. CD34^+ cells were selected according to the glycoprotease method of Sutherland et al. (1992) Exp. Hematol. 20:590-599. Briefly, cells were resuspended in staining buffer (SB) (HBSS containing 10 mM HEPES, 2% heat-inactivated FCS) at 5×10^7 cells/mL. QBEND10 (anti-CD34) (Amac, Westbrook, ME) was added at 1/100 dilution, and cells incubated on ice for 30 min. Cells were then washed in SB with a FCS underlay, and resuspended at 4×10^7 /mL in SB. An equal volume of washed Dynal sheep anti-mouse IgG₁Fc magnetic beads (Dynal, Oslo, Norway), was added at a 1:1 bead to

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- cell ratio, to give a final cell concentration of 2×10^7 cells/mL. After 30 min incubation on ice, with gentle inversion, the tube was placed against a Dynal magnet (Dynal) for 2 minutes, and CD34⁺ cells removed. Following
- 5 two washes, 20 μ L of 'glycoprotease' (O-sialoglycoprotein endopeptidase, Accurate Chemical, Westbury, New York) plus 180 μ L of RPMI (JRH Biosciences)/20% FCS were added and the beads incubated at 37°C for 30 min to cleave the QBEND10 epitope, and release CD34⁺ cells from the beads.
- 10 Beads were then washed three times to maximize cell recovery.

Adult Bone Marrow

- Fresh 20 mL adult bone marrow aspirates from normal donors were obtained from Stanford University or Scripps
- 15 Institute. Mononuclear cells were prepared by Ficoll density gradient centrifugation and subjected to CD34⁺ selection by the glycoprotease method described above. Alternatively, cadaveric bone marrow CD34⁺ cells were positively selected using a biotinylated anti-CD34
- 20 antibody (K6.1) and a biotin competition release according to the method described in PCT publication no. WO94/02016.

Example 2: FACS Purification of Primitive Hematopoietic Cells

- 25 CD15 depleted mononuclear cells (fetal) or CD34⁺ cells (adult) were fluorescently labeled using a two step procedure. All staining steps were performed on ice. Heat inactivated gamma globulin (Gamimmune, Miles Inc., Elkhart, IN) was added to the cell suspension at a
- 30 concentration of 1 mg/mL and incubated for 10 min. to block Fc receptor binding sites prior to adding antibody reagents. Cells were reacted with anti-CD34 (A. Ziegler,

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University of Berlin, Germany) or irrelevant mouse IgG3 (FLOPC21, Sigma, St. Louis, MO) at 7 μ g/mL for 20 min. The cells were washed once by diluting 20-fold with staining medium, underlaying with FBS, and pelleting at 5 700xg for 7 min. Cells were then reacted with goat anti-mouse IgG3 conjugated to Texas red (Southern Biotech Associates, Birmingham, AL) at a 1:100 dilution and a mixture of FITC-conjugated antibodies directed against a panel of lineage markers, the panel including anti-CD2, 10 anti-CD14, anti-CD15, anti-CD16, anti-CD19 (Becton Dickinson) and anti-glycophorin (Amac), all added at a 1:50 dilution. FITC-conjugated mouse IgG1, IgG2a, and IgM (Becton Dickinson) diluted 1:50 were used as controls for the lineage (lin) panel. Cells were washed once as 15 before and resuspended at a concentration of 10^6 cells/mL in staining medium containing 1 μ g/mL (PI) (Molecular Probes Inc., Eugene, OR).

Alternatively, in some experiments using adult cells, cells were stained using CD34 antibody (PR3) 20 directly conjugated to sulfarhodamine. In addition to the CD34/Lin staining, a two step Thy-1 (PR13) followed by goat anti-mouse IgG1-PE (Caltag) staining was performed, and the CD34⁺/Thy⁺/Lin⁻ population was sorted. In some cases anti-DC38-FITC (Amac) was used for negative 25 selection in place of the Lin panel. In these cases Thy-1 staining was omitted.

Fluorescently stained cells were analyzed on a FACStar+ cell sorter (Becton Dickinson, San Jose). Dead cells (PI positive) and cells positive for lineage 30 markers were excluded from the sort gate. Live lineage negative cells were gated for those expressing the highest amount of CD34 (a distinct population with mean fluorescence intensity greater than 100 times the isotype control values and representing approximately 4% of the 35 total CD15 depleted mononuclear cells). The CD34^{hi}Lin⁻

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cells were then deposited at one cell/well into Terasaki plates (Robbins Scientific, Mountain View) using an automated cell deposition unit (ACDU) program (Becton Dickinson, San Jose). The position of the cell stream
5 was preset by depositing a fluorescent bead into each well of a test plate prior to cell sorting. The accuracy of the single cell deposition was greater than 99%.

Example 3: Single Primitive Cell Culture

Terasaki plates used for single cell deposition were
10 preseeded with AC6.21 mouse stromal cells (see Baum et al. (1992) Proc. Natl. Acad. Sci. USA 89:2804-2808 copending United States patent application Serial No. 07/938,548, filed 8/28/92) at approximately 50 cells per well in 20 μ L AC6.21 culture medium (RPMI 1640, 5%
15 FBS, 10^{-5} 2-ME, 4 mM μ g/mL glutamine, 50 U/mL penicillin, and 50 μ g/mL streptomycin). Stromal cells were allowed to form a confluent monolayer by culturing for 3-7 days. Immediately prior to sorting, 50% of the medium (10 μ L) was removed and replaced with 10 μ L of stem cell culture
20 medium (SCCM) [IMDM (JRH Biosciences), 10% FBS, 10^{-5} M 2-ME (Sigma), 100 mM Na-pyruvate (JRH Biosciences), 4 mM glutamine (JRH Biosciences), 50 U/mL penicillin, and 50 μ g/mL streptomycin]. SCCM was supplemented with 50 μ g/mL 30% iron saturated human transferrin (JRH Biosciences)
25 and a cocktail of cytokines which included 10 ng/mL rhIL-3 (Genzyme), 10 ng/mL rhIL-6 (Sandoz), 20 ng/mL rhSLF (Tago/Biosource) and 10 ng/mL rhLIF (Tago/Biosource). (All concentrations are final concentrations in the SCCM.) Following single cell deposition, co-cultures
30 were maintained at 37°C in a humidified 5% CO₂ incubator and fed once per week by replacement of one half of the medium.

Scoring of Growth and Morphology. Each Terasaki well was examined microscopically at weekly intervals for

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10 weeks, after which time the hematopoietic cells had either expanded beyond the support capacity of the well or had terminally differentiated. The number and morphology of hematopoietic cells in each well was recorded. Morphology classifications were: (1) no growth; (2) blast cells (small round or tear drop-shaped refractile cells); (3) large granular cells (immediate precursors to macrophages); and (4) macrophages (terminal differentiation). Cell numbers ranged from 0 (cell death) to confluent (approximately 20,000 cells). Growth behaviors were sub-categorized depending on the rate at which a particular morphology developed.

Figure 1 outlines the visual (microscopic) analysis of hematopoietic proliferation after single cell plating. Some of the terms used in Figure 1 are defined as follows:

"Low cell production" is defined as appearance of 100 or fewer progeny of the input cell at any time. These are thought to be relatively mature progenitors which typically die out within a couple of weeks.

"Moderate cell production" refers to wells that generate more than about 100 progeny, but do not reach confluence or convert to macrophages. These display the capacity for production of more than 100 but fewer than 20,000 progeny cells in the culture system as described. In general they also show growth followed by rapid decline and detachment, but in some cases cells persist for many weeks without showing death or further noticeable expansion.

"Appearance of macrophages" is an end result in the differentiation of all wells showing high-level blast cell production: immature blast cells convert to large cells and then to macrophages. In some cases this occurs after secondary replating; in such a case the primary cell would be scored as producing a well reaching

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confluence without conversion. Whenever macrophages appear in a well with high blast cell production, a net decline in blast cell number is observed within 7-14 days. The macrophage categories are broken down by time of appearance of a significant number of cells with non-blast morphology (large, granular cells): wells with "late" appearance of macrophages generally have either a quiescent period before blast cell production, or early blast cell production that continues longer and with greater cell production before conversion. In either case, the data support a conclusion that late appearance implies a more primitive input cell.

"Confluent blast cells" arise from cells having the following characteristics: (1) proliferative potential that allows production of 20,000 cells with blast morphology; (2) lack of production of pre-macrophages during this period that would cause conversion to category (1); and (3) demonstration of quiescence, with variable term of quiescence ranging from zero to as much as ten weeks. The data obtained support the possibility that stem cells can be either quiescent or "activated" on input; confluence can be reached in three weeks or less starting with activated stem cells. A period of quiescence greater than one week followed by proliferation to confluence correlates highly with stem cell activity as measured by other factors, such as the detection of primitive cells after expansion or the production of lymphoid and myeloid cells after single-cell replating.

In addition to the weekly visual scoring, images were capture of each well three times weekly using a Universal Imaging (Westchester, PA) system. At the conclusion of an experiment, image series were reviewed. This allowed precise quantitation of cell numbers for

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generation of growth curves, quantitation of total cell production, and documentation of growth patterns.

Example 4: Phenotypic Analysis of Confluent Wells

Wells which became confluent with blast cells were phenotypically analyzed by FACS for the number of primitive progenitors (CD34⁺Lin⁻), cells in the myeloid lineage (CD33⁺), and cells in the B lymphoid lineage (CD19⁺, CD10⁺). Standard methods known in the art were used to stain and sort the cells. Briefly, hematopoietic cells were removed from the confluent wells by gentle pipeting. The contents of each well were divided into two samples, each containing approximately 10⁴ cells and resuspended in 100 μ L staining medium. Samples were stained with either anti-CD34 plus the panel of lineage antibodies or anti-CD33 plus anti-CD19 and anti-CD10 using a scaled-down procedure similar to that used for the primary staining. A pool made from a small aliquot from each of the wells was used for control Ig staining.

Figures 2 and 3 categorize confluent wells by the time needed to reach confluence and correlate the data with the phenotype of expanded cells for two experiments, each with different tissues. These data demonstrate a number of important points.

First, wells reaching confluence with blast cells, an indicator of primitiveness and proliferative potential, can be further analyzed for phenotype.

Second, some input cells have the capability to reach confluence and retain a measurable (2% above FACS background in the sorted gate) primitive population of CD34⁺Lin⁻ cells in the progeny. This threshold translates to roughly 400 progeny cells in this category, or a 400-fold expansion of the CD34⁺Lin⁻ compartment from the starting single cell. This result correlates very highly

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with primitive stem cell phenotype and behavior, because only a very primitive cell will have the capability of making large numbers of primitive cells.

Third, some input cells have the capability of
5 producing both lymphoid (CD19⁺ or CD10⁺) cells and myeloid cells (CD33⁺). Myeloid cells are present in virtually all blast cell expansions performed under these conditions. The display of multilineage potential is an important indicator of the primitive character of the
10 input cell. In some cases, single input cells that appear to be stem cells by other criteria (e.g., production of >20,000 progeny cells or retention of CD34⁺Lin⁻ cells after expansion) fail to produce measurable amounts of B cells. This is thought to
15 reflect a possible predisposition of stem cells to myeloid commitment in the culture system described; if the early differentiation steps of a stem cell all lead to myeloid commitment, then B cells will not be observed.

Figure 4 shows examples of FACS analysis of single
20 CD34^{hi}Lin⁻ cells from fetal bone marrow SF268 which expanded to confluence. Plot #1 shows a portion of the cultured cells stained with the isotype control antibodies mouse IgG1-PE (Y axis) plus IgG1-FITC (X axis). Plots #2 and #3 show the contents of individual
25 wells stained with anti-CD33-PE (Y axis) and anti-CD19-FITC and anti-CD10-FITC (X axis) to test for the production of both myeloid and B lymphoid lineages from one cell. Plot #2 is an example of a well that contained a population of myeloid cells and a population of cells
30 that stained for neither the myeloid or B lymphoid markers. Plot #3 is an example of a well which contained a myeloid, B lymphoid. Plots #4, #5, #6, and #7 are examples of individual wells stained with anti-CD34 plus goat anti-mouse IgG3-Texas red (Y axis) and anti-Lin-FITC
35 (X-axis) to test for the maintenance and expansion of

-42-

primitive progenitors. Plot #4 shows the isotype control staining. Plot #5 shows a well which contained primarily lineage positive cells. Plots #6 and #7 show wells which contained a substantial CD34⁺Lin⁻ expanded population
5 (approximately 7,000-9,000-fold expansion from one cell).

Example 5: Secondary expansions

If a well was found to contain a population of cells with a CD34⁺Lin⁻ phenotype which was greater than 2% of the total cells in the well, this population was resorted
10 at one cell per well into secondary plates. The procedure for culturing, scoring, and analysis was the same as for primary plates.

The data from secondary replating show dramatic enrichment of certain cell types and behaviors. Figure 5
15 presents FACS analyses showing the remarkably primitive behavior in culture of a cell from the CD34⁺Lin⁻ population from human fetal bone marrow SF293. Following Ficoll gradient separation and anti-CD15 bead depletion, the CD34⁺Lin⁻ population was sorted at one cell per well
20 into a 72 well Terasaki plate. Figure 5A shows the population selected for plating (inside R2). CD34 staining is shown on the Y axis. Also shown is the reanalysis of the sorted population: after Terasaki plating, the remaining unsorted cells were sorted in bulk
25 using the same gates; then the bulk sort was reanalyzed. Each Terasaki well had been preseeded with a AC6.21 stromal layer and contained nutritive media supplemented with 10 ng/mL rhIL-3, rhIL-6, rhLIF, and 25 µg/mL rhSLF. Over the course of ten weeks in culture, 16/72 (22%) of
30 the wells reached confluence. Upon FACS analysis, six of these were found to contain an expanded CD34⁺Lin⁻ population ranging from 3-18% of the total cells in the well.

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One well (#9) reached confluence by week four of culture and contained 18% CD34⁺Lin⁻ cells out of approximately 20,000 cells, or roughly 3,600 expanded primitive cells. This analysis is shown in Figure 5B (CD34 staining is on the Y axis and Lin staining is on the X axis). The isotype control staining is shown for comparison. 45 cells from this population were replated as single cells in secondary culture.

Two wells (4.4%) from the secondary plating reached confluence at 4 and 5 weeks after secondary plating, respectively. The well reaching confluence at week 4 consisted almost entirely of myeloid cells (Figure 5C, panel 1). The well reaching confluence at 5 weeks contained a CD34⁺Lin⁻ population of 5%, a B lymphoid population of 3%, and a myeloid population of 8% (Figure 5C, panel 2). Isotype control staining is shown in Figure 5C, panel 3. These results demonstrate that the original input cell was sufficiently primitive to maintain its phenotype through nine weeks of culture and give rise to a primitive population as well as to both B lymphoid and myeloid populations.

The secondary expansion data presented in Figure 6 relate to a series of secondary cultures of the CD34⁺Lin⁻ portions of expanded single cells reaching confluence at 0-17 days (Figure 6A), by 28 days (Figure 6B), between 39 and 45 days (Figure 6C), and after 42 days (Figure 6D). These data show dramatic enrichment of certain cell types and behaviors, accomplished by primary single cell expansion followed by immunophenotype sorting. For example, for well 2E10, derived from primary single cell expansion, over 80% of the cells which expanded after sorting and secondary plating converted to macrophages within 17 days. Well 2D4 shows secondary expansion to confluence in 20% of CD34⁺Lin⁻ replated single cells, indicating again the very primitive nature of the single

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primary input cell. In each case, the relative homogeneity of behavior (and presumably of identity of secondary input cells) is remarkable for a population of cultured primitive hematopoietic cells.

5 Example 6: PKH26 Dye (Zynaxis) Labeling

In experiments with dye labelled cells, labelling was performed following CD34 positive selection and before fluorescent antibody labeling. Dye labeling was performed per manufacturer's instructions (Sigma) with
10 the following modifications. Due to the small number of cells being labeled (approximately one million cells are commonly recovered after a CD34 positive selection of a 20 mL adult bone marrow aspirate) the volume of the final labeling solution was 1 mL instead of 2 mL.

15 PKH26 fluoresces strongly in the PE (FL2) channel. Therefore, in sorts using dye labeled cells Thy-PE could not be used for positive selection. For these experiments a CD34-SR positive, CD38-FITC negative population was sorted. In addition, a dye intensity gate
20 was defined such that the sorted population would not vary by more than two fold in PKH26 staining intensity. Typically, the intensity of staining varied from 1,000 to 10,000 and a gate from 4,000 to 8,000 was selected. This allowed more precise tracking of division number.

25 Example 7: Study of Quiescence vs. Potency of Progenitors After In Vitro Expansion

The adult hematopoietic cell samples were debulked, CD34 positive selected, PKH26 dye labeled, and sorted as described above. The sorted CD34⁺/CD38⁻ or CD34⁺/Thy⁺/Lin⁻
30 populations were plated at 10⁴ cells in 1 mL in a 24 well plate using the same medium and cytokine conditions described for single cell plating. These bulk cultures were grown either on or not on a AC6.21 stromal layer.

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Bulk cultures were carried for 16 days with weekly replacements of one half of the culture medium. At 16 days cells had expanded 30-120 fold in number. The cultured cells were stained with anti-CD34 and the lineage panel, and two populations were sorted: (1) CD34⁺/Lin⁻/dye bright; (2) CD34⁺/Lin⁻/dye dim. Based on the dye intensity of the starting population (before bulk culture), the dye bright progenitors had divided (reduced their dye intensity by half) four or less times. The dye dim progenitors had divided more than four times (see Fig. 8 for strategy for sorting these two populations). These two populations were sorted using ACDU into Terasaki plates and the single cell assay method was carried out as described in the existing patent (see Fig. 7 for a flow chart of the experimental design).

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications cited herein are hereby incorporated herein by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

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What is claimed is:

1. A method of identifying hematopoietic stem cell activity comprising the steps of:
 plating a single input hematopoietic cell in an
5 appropriate culture system;
 culturing the input cell under conditions which allow it to expand to produce progeny cells; and
 analyzing the number and phenotype of the progeny cells, wherein the input cell is scored as a stem cell if
10 the input cell possesses the ability to proliferate from a single cell to approximately 8,000 progeny cells or more.
2. The method according to claim 1 wherein the input hematopoietic cell is derived from a fetal source and the
15 progeny cells proliferate to 20,000 cells or more.
3. The method according to claim 1 wherein the input cell is additionally scored as a stem cell if the input cell possesses the potential to produce both myeloid and lymphoid progeny cells.
- 20 4. The method according to claim 3 wherein the input cell is additionally scored as a stem cell if cells with a primitive immunophenotype are retained after expansion of the input cell.
5. The method according to claim 3 wherein the input
25 cell is additionally scored as a stem cell if cells with a primitive proliferative behavior are retained after expansion of the input cell.
6. The method according to claim 1 wherein the culture system comprises stromal co-culture.

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7. The method according to claim 6 wherein the stromal co-culture is a AC6.21 stromal co-culture.
8. The method according to claim 1 wherein the culture system further comprises effective amounts of IL-3, IL-6,
5 SLF, and LIF.
9. The method according to claim 8 wherein the culture system further comprises effective amounts of at least one member of the group consisting of: IL-1, IL-7, IL-11, G-CSF, GM-CSF, and an IL-3/GM-CSF fusion protein.
- 10 10. The method according to claim 1 wherein analysis of the proliferation or multipotency or both of the input cell occurs when the input cell expands to Terasaki well confluence.
11. The method according to claim 10 wherein analysis of
15 the input cell occurs when the input cell expands to confluence in a Terasaki well.
12. The method according to claim 1 wherein the progeny cells are analyzed by fluorescence activated cell sorting after expansion of the input cell.
- 20 13. The method according to claim 1 further comprising the steps of:
 selecting a progeny cell;
 replating the selected progeny cell in an appropriate culture system; and
25 incubating the replated progeny cell under conditions sufficient to allow expansion.
14. The method according to claim 13 wherein said selected progeny cell is replated as a single cell.

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15. The method according to claim 13 wherein selection is accomplished by fluorescence activated cell sorting.

16. A method of identifying hematopoietic stem cell activity comprising the steps of:

- 5 plating a single input hematopoietic cell in an appropriate culture system;
 culturing the input cell under conditions which allow it to expand to produce progeny cells; and
 analyzing the number and phenotype of the progeny
10 cells, wherein the input cell is scored as a stem cell if the input cell displays a dispersed growth pattern upon cell culture.

17. A method of assaying hematopoietic stem cell activity comprising the steps of:

- 15 plating a single input hematopoietic cell in an appropriate stromal co-culture system comprising effective amounts of at least one of the cytokines IL-3, IL-6, SLF, and LIF;
 incubating the input cell under conditions
20 sufficient to allow expansion to confluence or until approximately 8,000 progeny cells are produced or both;
 analyzing the number and phenotype of the progeny cells; and
 scoring the input cell as a stem cell if the input
25 cell possesses the ability to proliferate from a single cell to approximately 8,000 cells or more.

18. The method according to claim 17 wherein the input hematopoietic cell is derived from a fetal source and the progeny cells proliferate to 20,000 cells or more.

- 30 19. The method according to claim 17 wherein the input cell is additionally scored as a stem cell if, the input

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cell possesses the potential to produce both myeloid and lymphoid progeny cells.

20. The method according to claim 19 wherein the input cell is additionally scored as a stem cell if cells with
5 a primitive immunophenotype are retained after expansion of the input cell.

21. The method according to claim 20 wherein the input cell is additionally scored as a stem cell if cells with a primitive proliferative behavior are retained after
10 expansion of the input cell.

22. The method according to claim 17 further comprising the steps of:
selecting a progeny cell;
replating the selected progeny cell in an
15 appropriate culture system; and
incubating the replated progeny cell under conditions sufficient to allow expansion.

23. A method of assaying hematopoietic stem cell activity comprising the steps of:
20 plating a single input hematopoietic cell in an appropriate stromal co-culture system comprising the cytokines IL-3, IL-6, SLF, and LIF;
incubating the input cell under conditions sufficient to allow expansion to confluence or until
25 approximately 8,000 progeny cells are produced or both;
analyzing the number and phenotype of the progeny cells;
selecting a progeny cell;
replating the selected progeny cell in an
30 appropriate culture system;

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- incubating the replated progeny cell under conditions sufficient to allow expansion; and scoring the input cell as a stem cell if
- (1) the input cell possesses the ability to proliferate from a single cell to approximately 8,000 cells or more;
- (2) the input cell possesses the potential to produce both myeloid and lymphoid progeny cells; and (3) cells with a primitive immunophenotype are retained after expansion of the input cell.
- 10 24. The method according to claim 23 wherein the input hematopoietic cell is derived from a fetal source and the progeny cells proliferate to 20,000 cells or more.
- 15 25. An isolated hematopoietic stem cell identified by the method of claim 1 and capable of producing at least 2 x 10⁶ progeny cells.
26. A method for obtaining a desired hematopoietic cell type comprising the steps of:
- 20 plating a single input hematopoietic cell in a stromal co-culture system comprising an effective amount of at least one of the cytokines IL-3, IL-6, SLF, and LIF;
- 25 culturing the input cell under conditions which all progeny cells are produced or both;
- analyzing the number and phenotype of the progeny cells by fluorescence activated cell sorting; and selecting a progeny cell of the desired hematopoietic cell type.
- 30 27. The method according to claim 26 wherein the input hematopoietic cell is derived from a fetal source and the progeny cells proliferate to 20,000 cells or more.

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28. The method according to claim 26 further comprising the steps of:

replating the selected progeny cell in an appropriate culture system; and

5 incubating the selected progeny cell under conditions sufficient to allow expansion.

29. The method according to claim 27 wherein the selected progeny cell is replated as a single cell.

30. The method according to claim 26 wherein the
10 selection is accomplished by fluorescence activated cell sorting, multiple progeny cells are selected, and the selected progeny cells are replated in bulk in an appropriate culture system.

31. The method according to claim 26 wherein the desired
15 hematopoietic cell type is a hematopoietic stem cell.

32. The method according to claim 31 wherein the steps of the method are repeated in cycle, multiple progeny cells are selected, and the selected progeny cells are replated in bulk in an appropriate culture system, thus
20 producing with each cycle an increased number of hematopoietic stem cells.

33. The method according to claim 32 wherein
the selection is accomplished in one or more initial cycles by fluorescence activated cell sorting and
25 the selection in one or more later cycles is accomplished by a method, other than fluorescence activated cell sorting, based on immunoaffinity or cell size or density.

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34. The method according to claim 33 wherein the culture system is a bioreactor.
35. A hematopoietic cell produced by the method according to claim 26.
- 5 36. A method for evaluating a sample for the presence of a substance affecting a biological response of a hematopoietic cell, said method comprising the steps of:
 plating a hematopoietic cell produced by the method
 of claim 26 or a progeny thereof as a test cell in an
10 appropriate culture system along with the sample;
 plating a hematopoietic cell produced according to
 the method of claim 26 or a progeny thereof as a control
 cell in an appropriate culture system without said
 sample; and
15 comparing the biological response of the test and
 control cells.
37. The method according to claim 36 wherein the test and control hematopoietic cells are hematopoietic stem cells.
- 20 38. The method according to claim 36 wherein said test and control hematopoietic cells are allowed to expand to produce progeny cells and said comparison comprises determining the number and phenotype of the progeny cells.
- 25 39. A method of treating an animal affected by a genetic disease comprising introducing into said animal a hematopoietic cell produced by the method according to claim 26 or progeny thereof, wherein the hematopoietic stem cell or progeny thereof is transfected with a
30 recombinant nucleic acid capable of either expressing in

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said transfected cell a polypeptide which is missing or defective in said animal or a expressing a nucleic acid or polypeptide capable of inhibiting the expression of a target protein in said animal.

- 5 40. A method of monitoring stem cell activity during the course of a medical treatment comprising the steps of:
- determining stem cell activity of a hematopoietic cell sample of a patient before the commencement of the treatment by the method according to claim 1, thereby
- 10 obtaining a baseline level of stem cell activity; determining the stem cell activity of a hematopoietic cell sample of a patient during the course of the treatment by the method according to claim 1, thereby obtaining a test level of stem cell activity; and
- 15 comparing the baseline and test levels.

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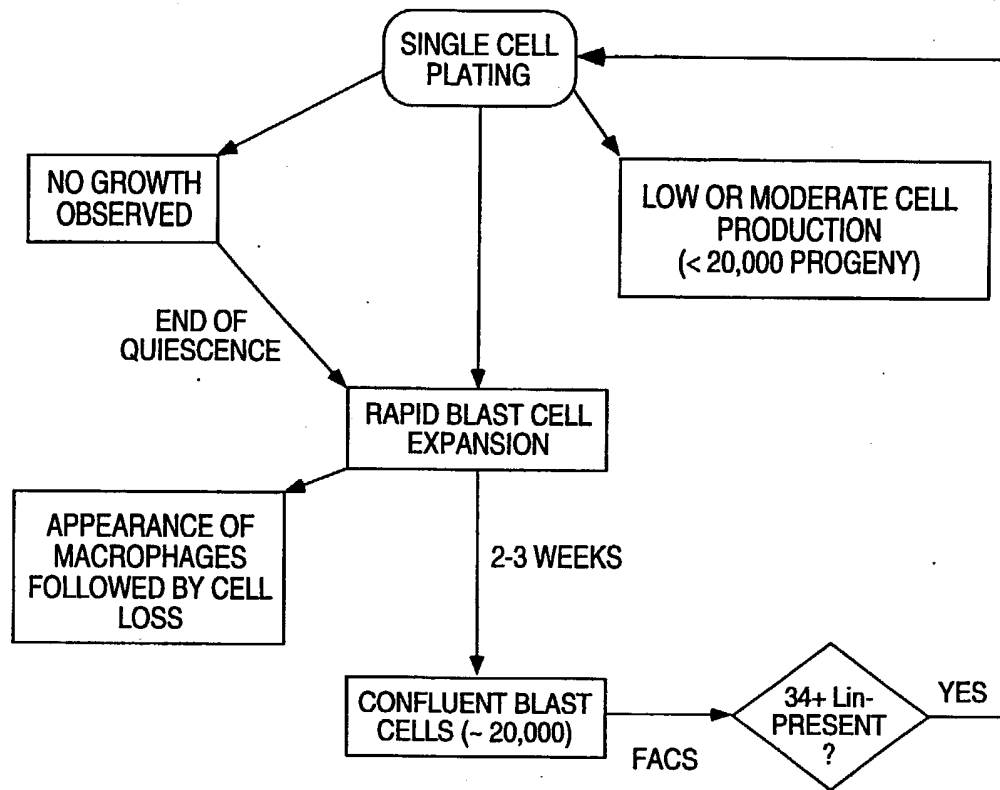


FIG. 1

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<u>WELL #</u>	<u>DAYS TO CONFL.</u>	<u>%34+ LIN-</u>	<u>%19+10</u>	<u>2NDARY PLT. BLAST CELL MAINT. (WEEKS)</u>
1D1	0-17	15		2
2E10				
2D5				
2D8				
2C10				
2C12				
2A5	4	4		3
2C2				
n=8			% WELLS WITH 34+, LIN- % WELLS WITH B CELLS	25% 0%
<hr/>				
1F5	18-31	15	10	3
1E9				
2E1				
2E8				
2E12				
2B1				
2B5				
2B6				
n=8			% WELLS WITH 34+, LIN- % WELLS WITH B CELLS	50% 38%
<hr/>				
2D7	32-38	20	2	
2B8			72	
n=2			% WELLS WITH 34+, LIN- % WELLS WITH B CELLS	50% 100%
<hr/>				
1B12	39-45	46		3
2D4		31		4
2D1				
n=3			% WELLS WITH 34+, LIN- % WELLS WITH B CELLS	67% 0%

FIG. 2

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WELL	DAYS TO CONFL.	POP	GF	%34+, LIN-	%19+10
A2	0-21	34LO	36SL		
B2		34LO	36SL		
E7		34HI	36SL		
C10		34HI	36SL	10.0	
B1		34HI	36SL		
E4		34HI	6L		
B6		34HI	6L		
n=7				% WELLS WITH 34+, LIN-	14%
				% WELLS WITH B CELLS	0%
F3	22-28	34HI	36SL	2.7	
F9		34HI	36SL	18.0	
F10		34HI	36SL		
F11		34HI	36SL		
E1		34HI	36SL		
B7		34HI	36SL		
A7		34HI	36SL	10.0	
C7		34HI	6L	1.5	
B9		34HI	6L		
E5		34LO	6L		
A4		34LO	6L		
n=11				% WELLS WITH 34+, LIN-	36%
				% WELLS WITH B CELLS	0%
A1	29-42	34HI	36SL		
D1		34HI	6L	5.0	
D11		34HI	6L		
A4		34HI	6L		
A4		34HI	6L		
n=5				% WELLS WITH 34+, LIN-	20%
				% WELLS WITH B CELLS	0%
A8	43-49	34HI	36SL		
A11		34HI	36SL	11.0	
n=2				% WELLS WITH 34+, LIN-	50%
				% WELLS WITH B CELLS	0%
C2	50-63	34LO	6L	2.5	
F7		34HI	36SL		
B10		34HI	36SL	8.3	
n=3				% WELLS WITH 34+, LIN-	67%
				% WELLS WITH B CELLS	0%
F1	64-77	34HI	36SL		
n=1				% WELLS WITH 34+, LIN-	0%
				% WELLS WITH B CELLS	0%

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FIG. 3

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FIG. 4A

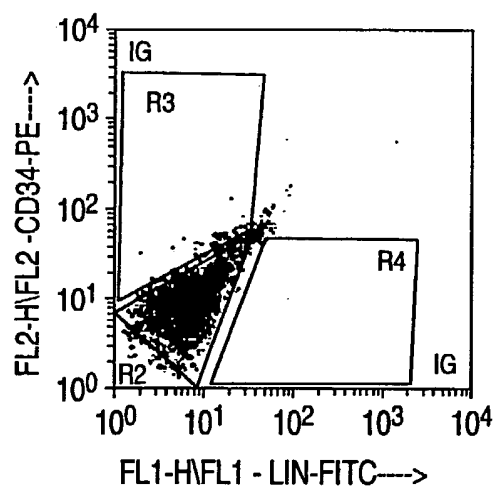


FIG. 4B

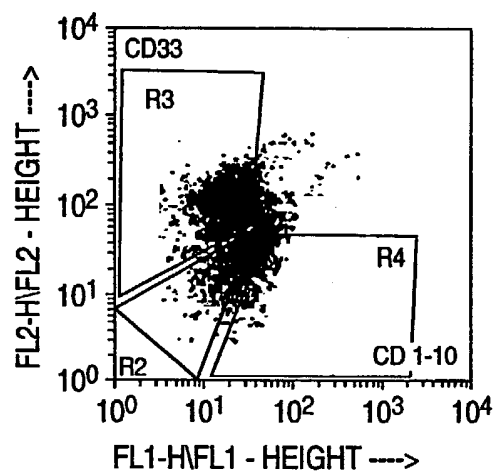
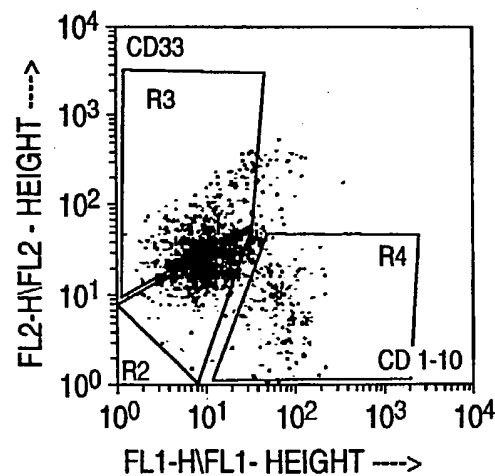


FIG. 4C



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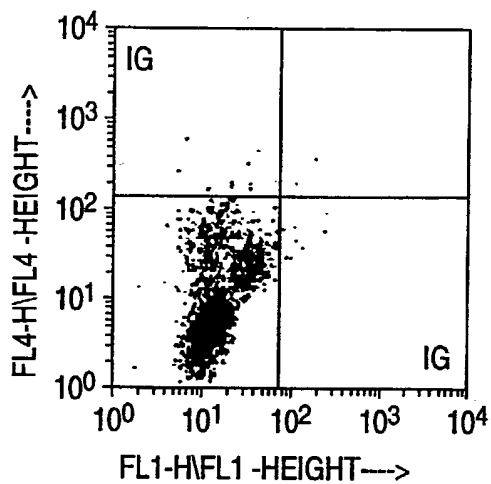


FIG. 4D

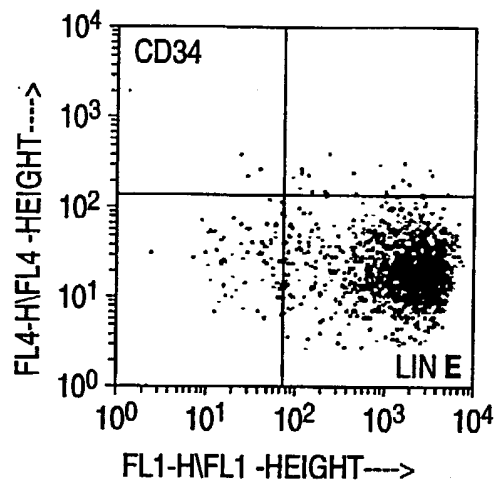


FIG. 4E

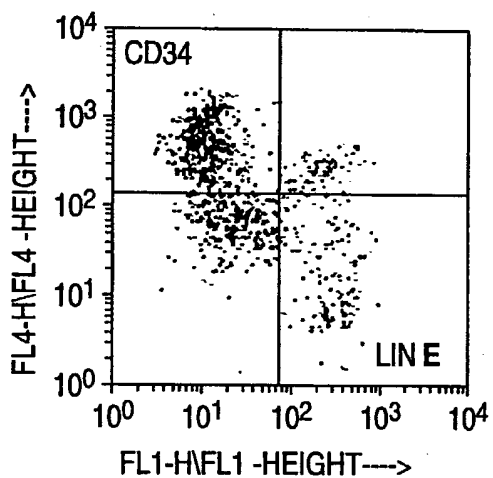


FIG. 4F

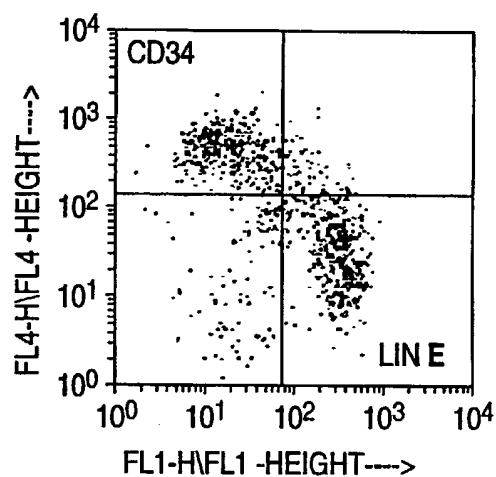
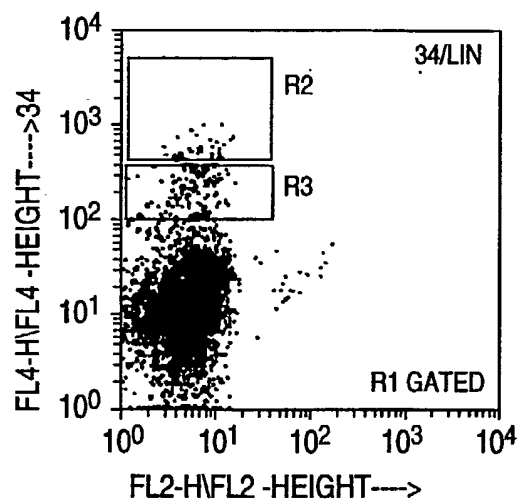
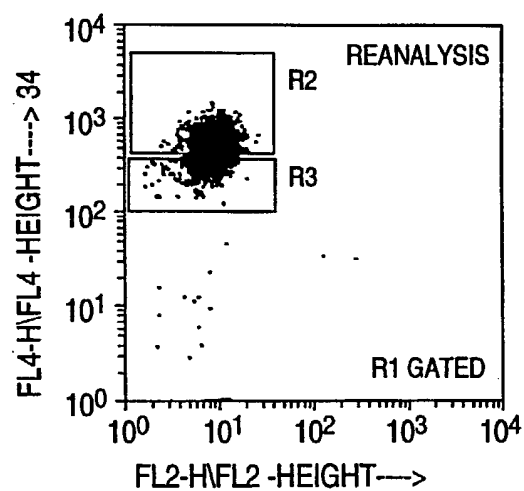


FIG. 4G

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**FIG. 5A****FIG. 5B**

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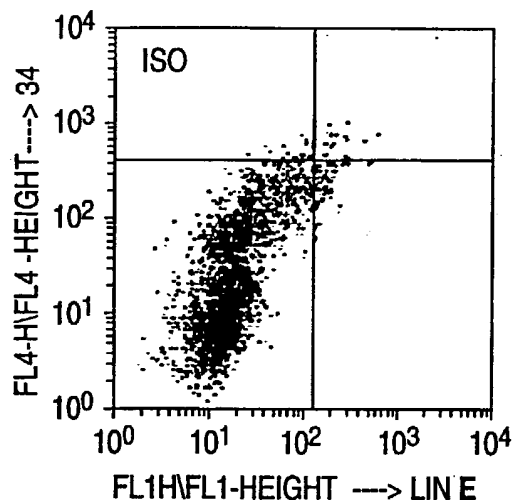


FIG. 5C

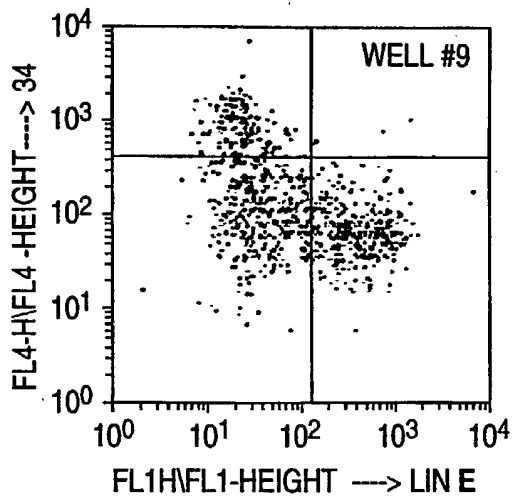


FIG. 5D

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FIG. 5E

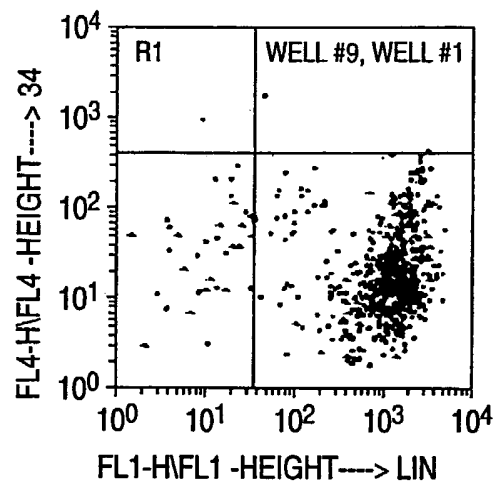


FIG. 5F

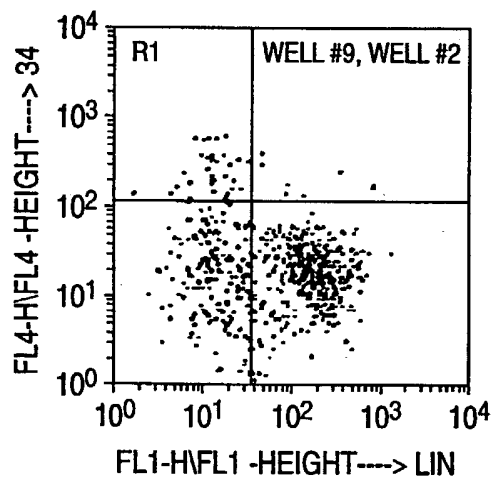
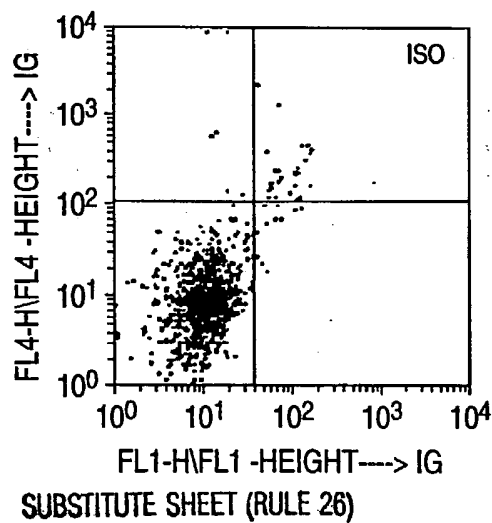


FIG. 5G



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FIG. 5H

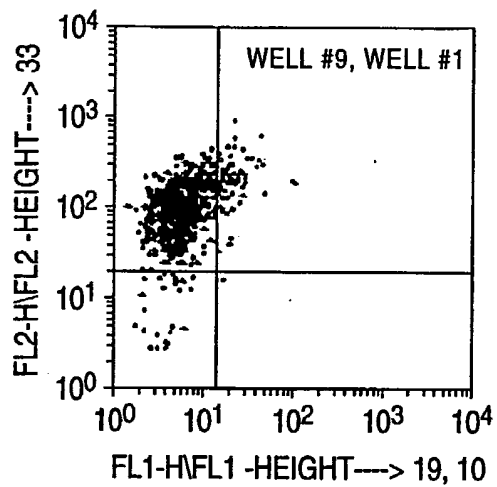


FIG. 5I

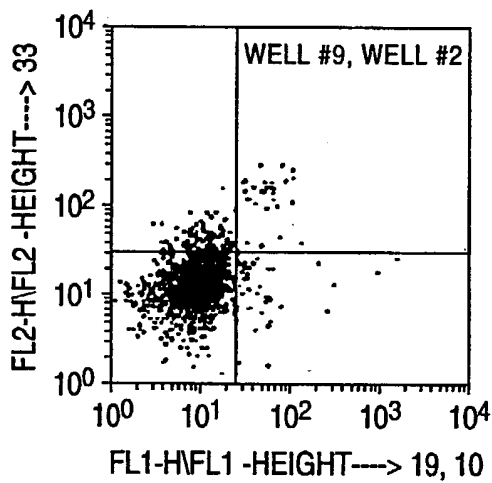
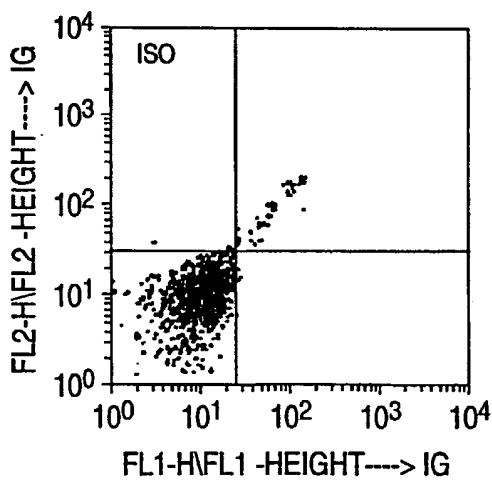


FIG. 5J



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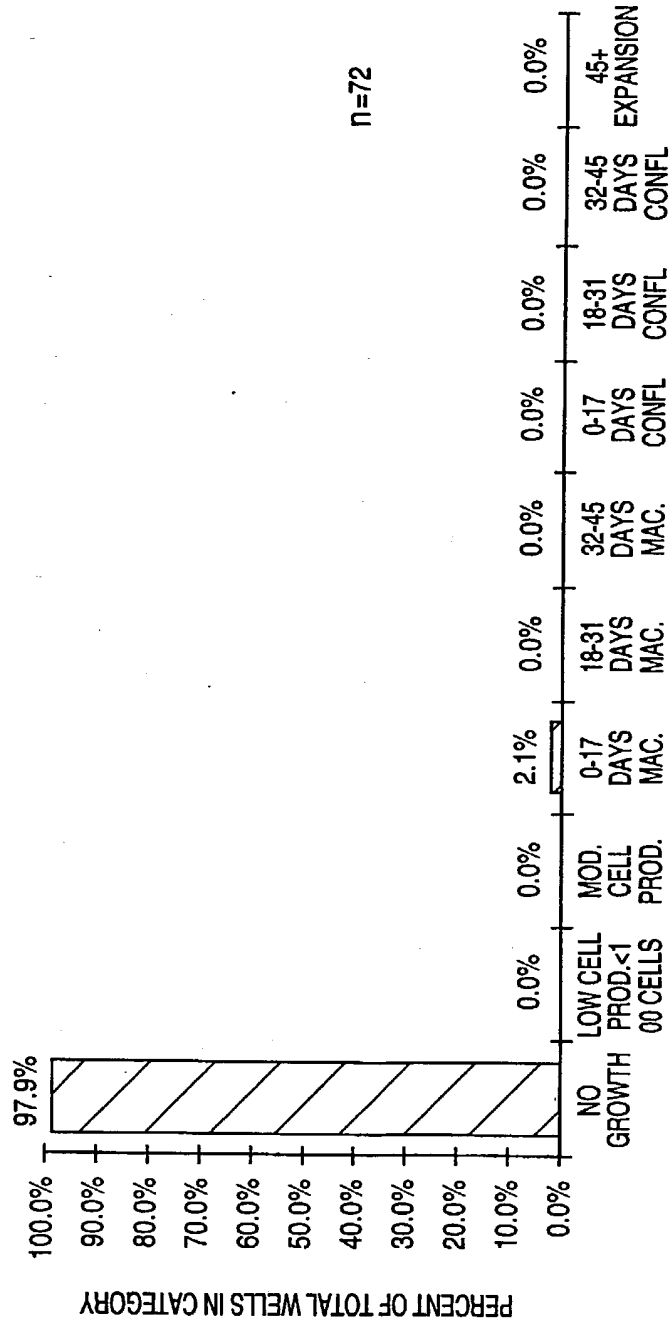


FIG. 6A

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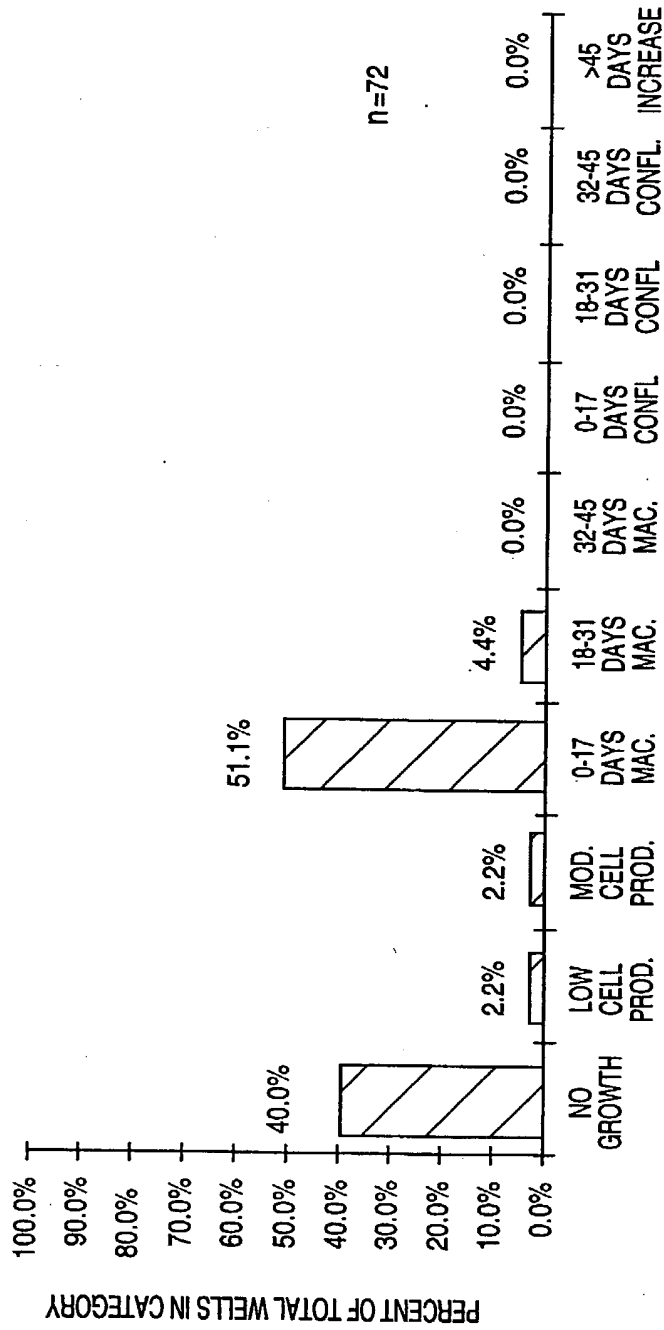


FIG. 6B

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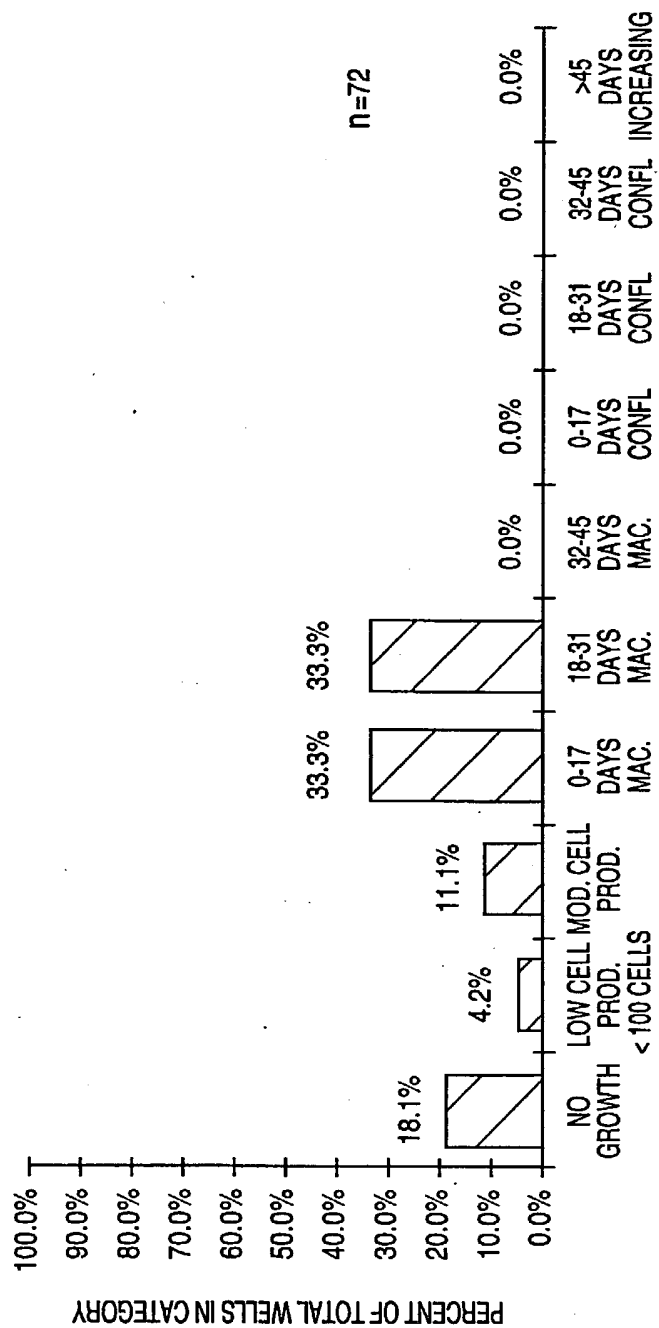


FIG. 6C

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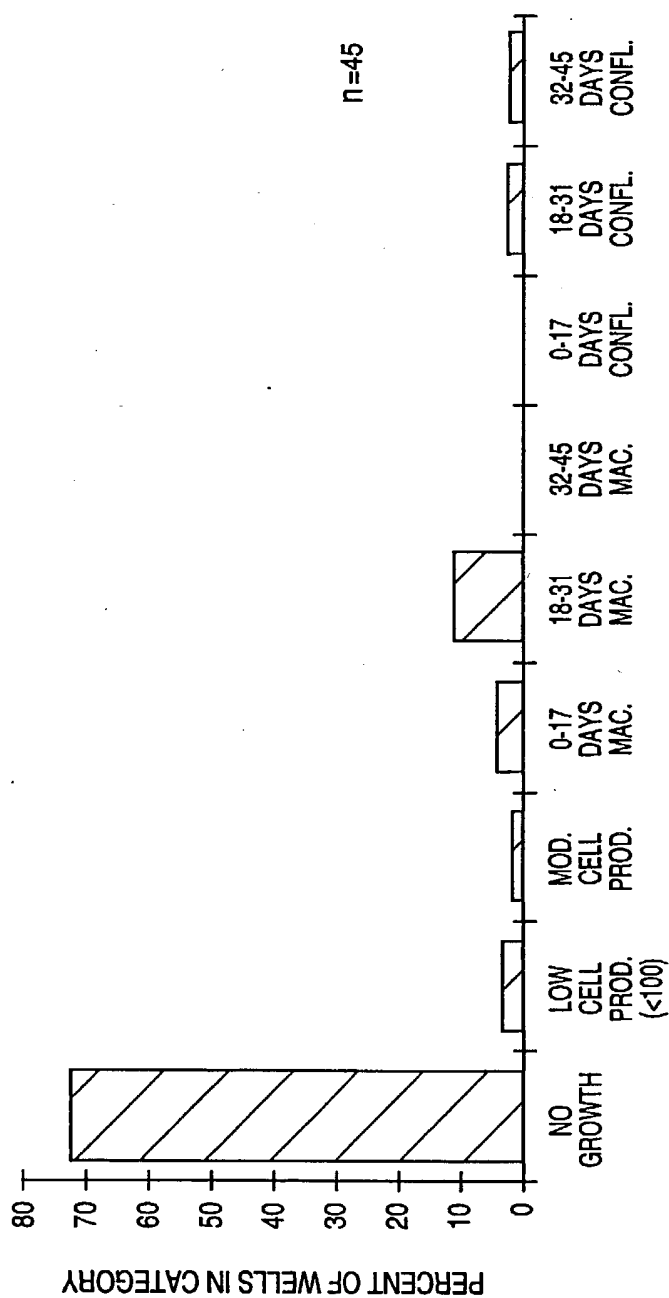


FIG. 6D

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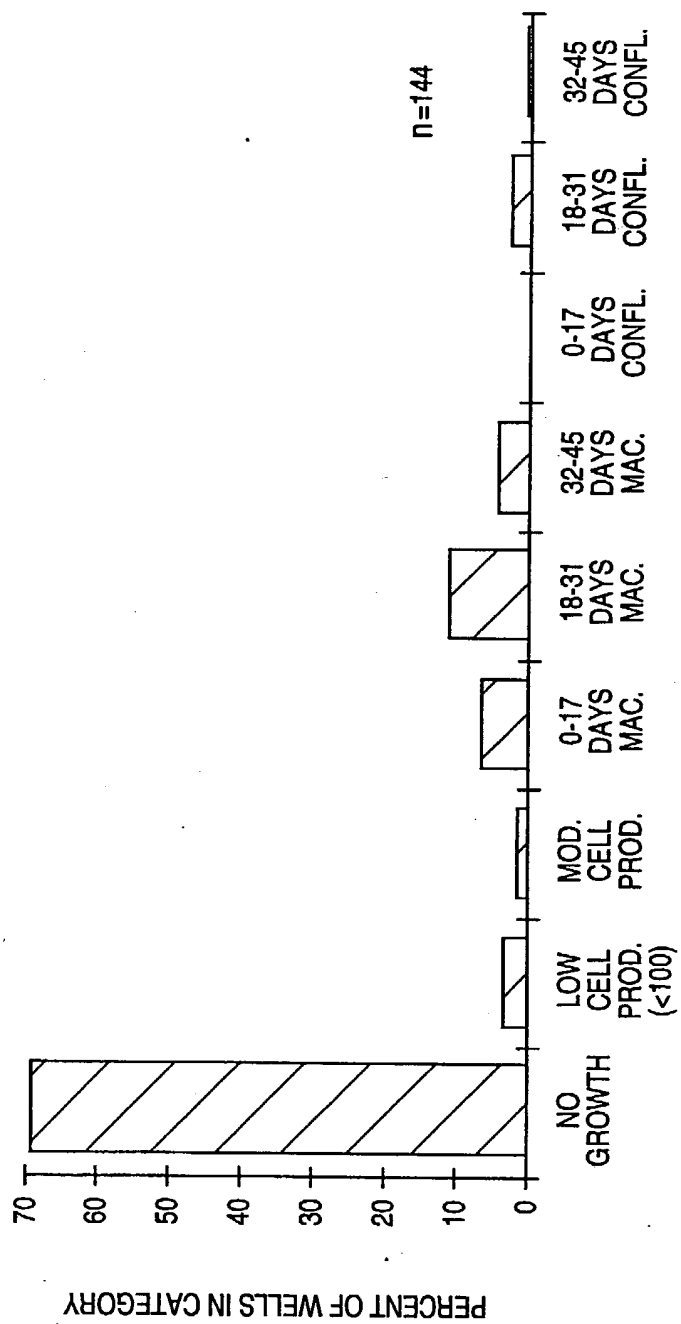


FIG. 6E

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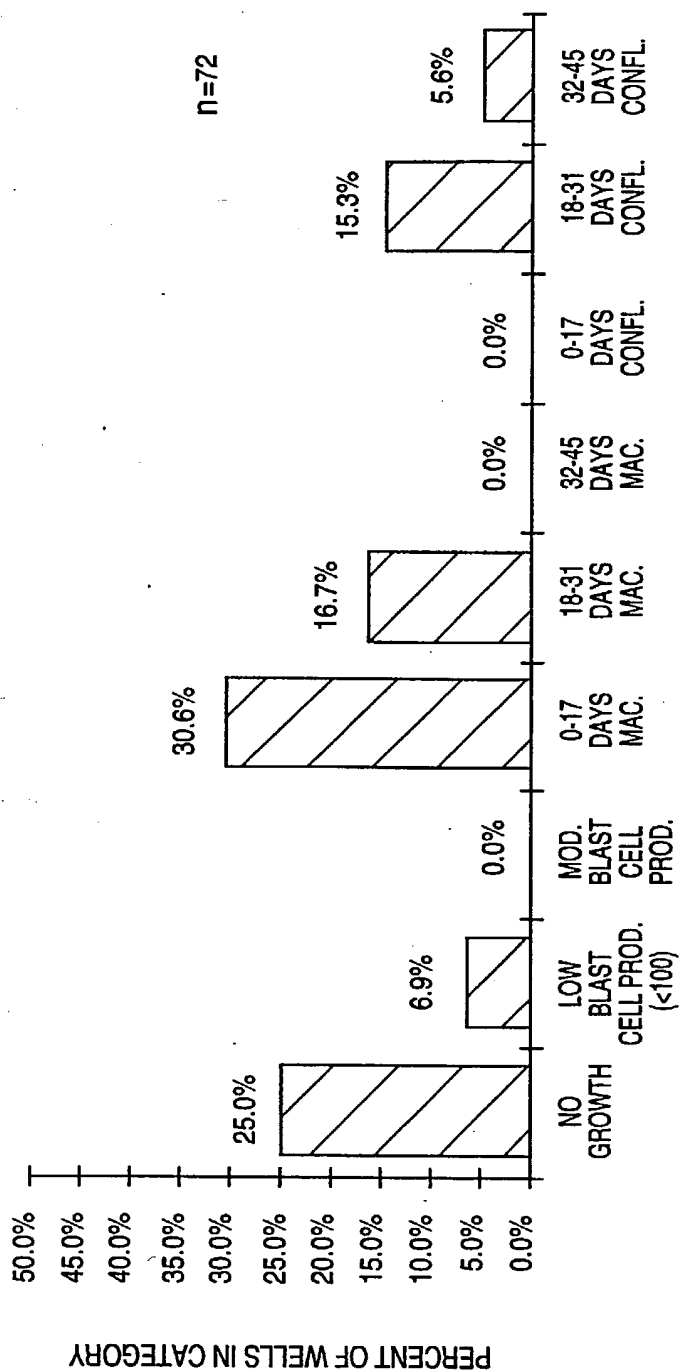


FIG. 6F

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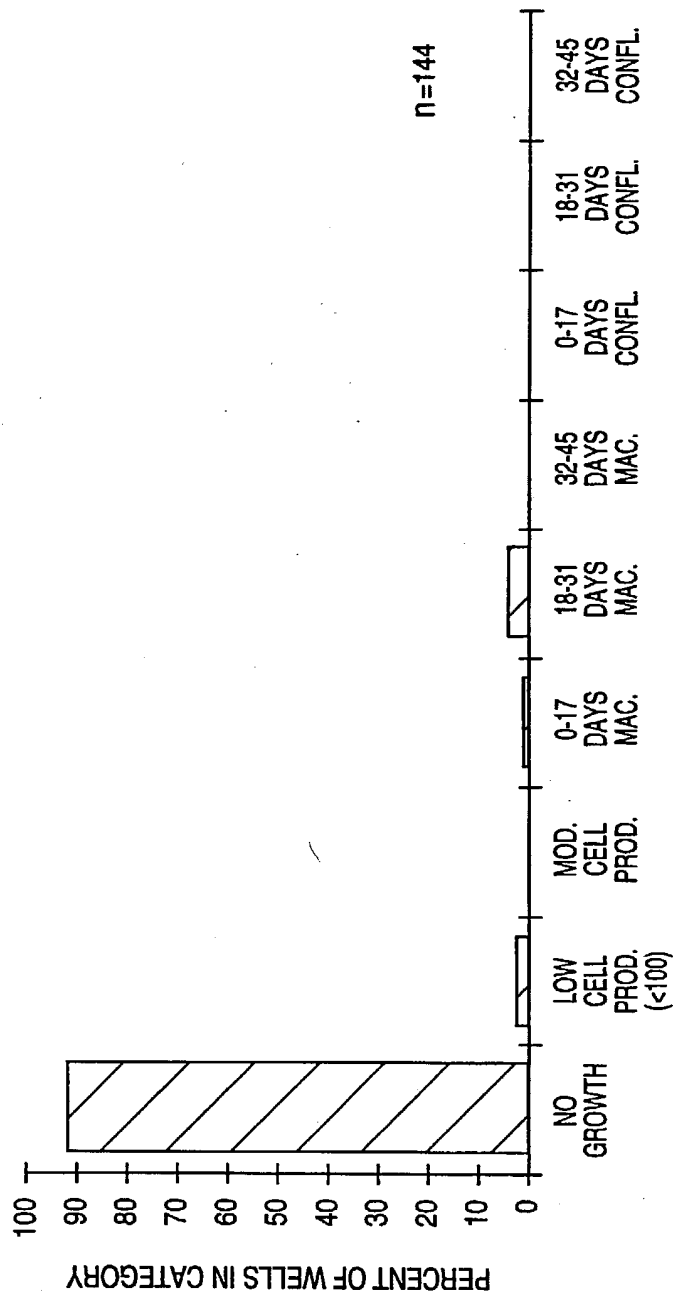


FIG. 6G

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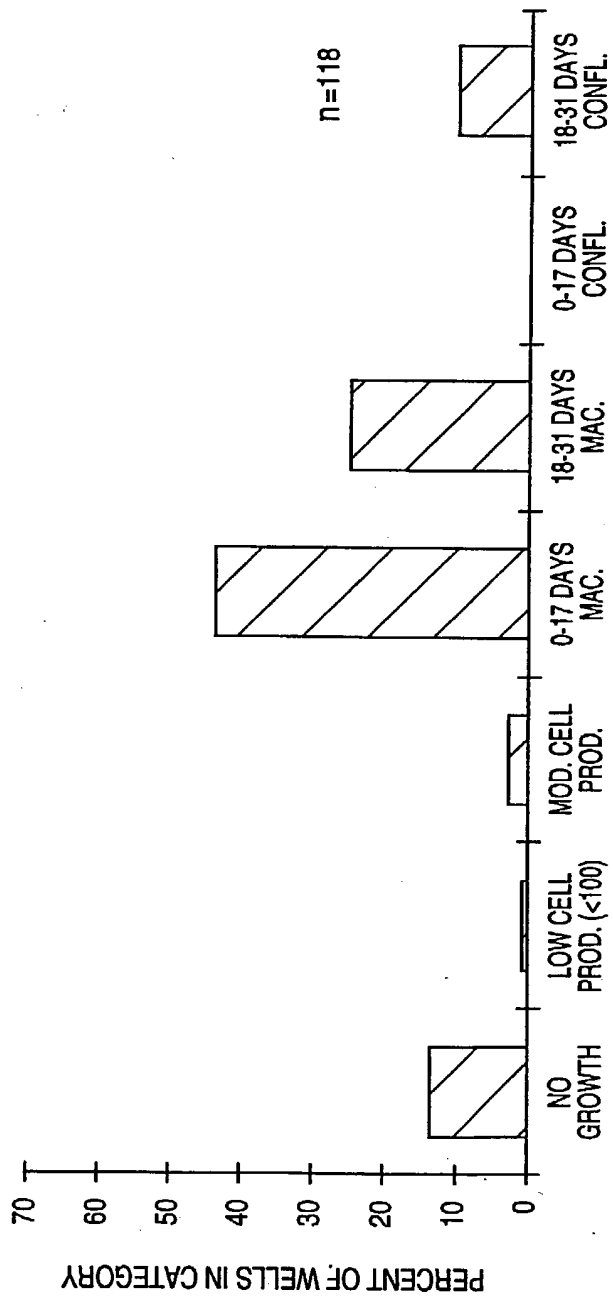


FIG. 6H

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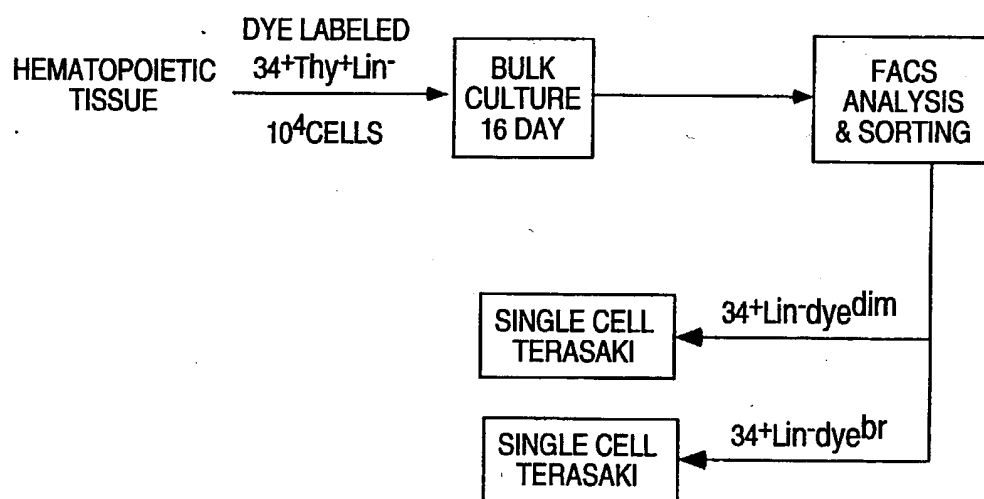


FIG. 7

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FIG. 8A

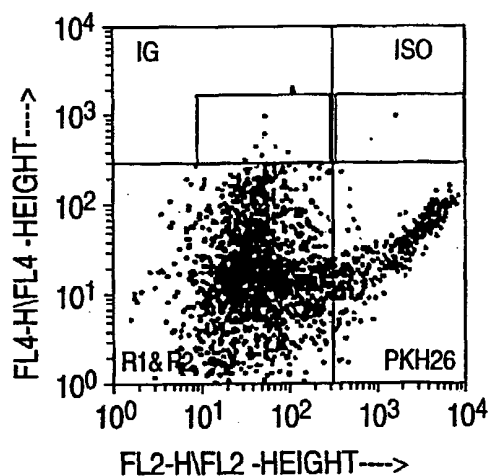


FIG. 8B

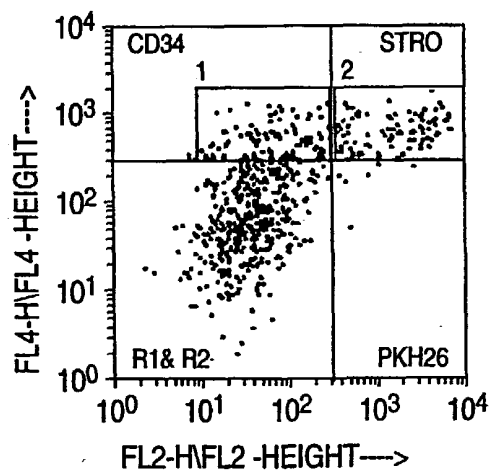
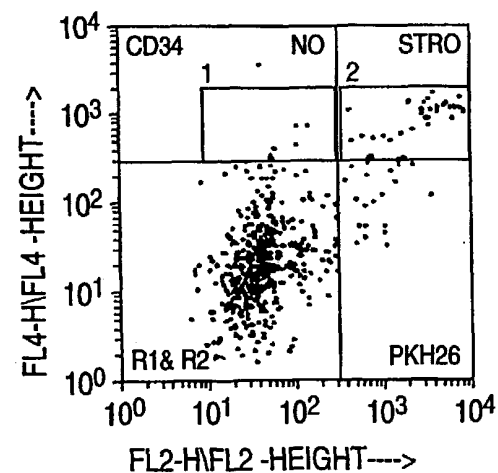


FIG. 8C



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FIG. 8D

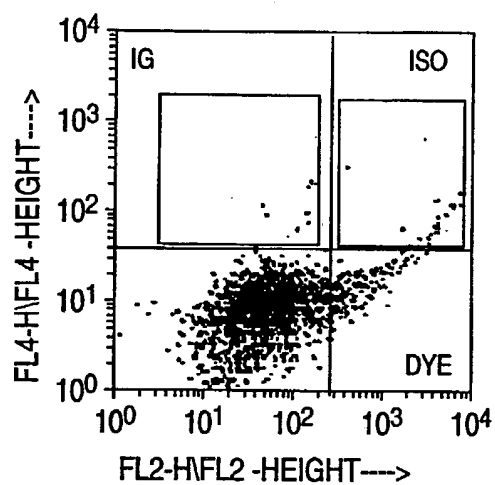


FIG. 8E

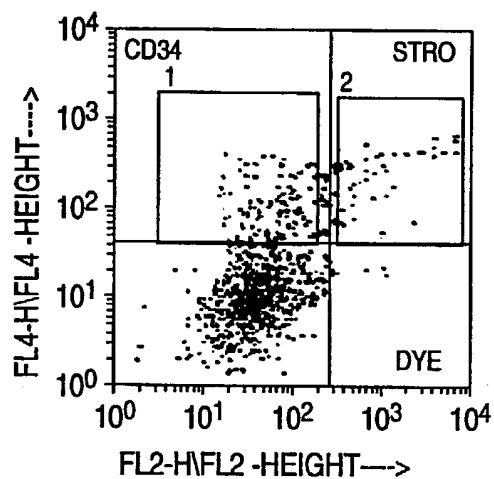
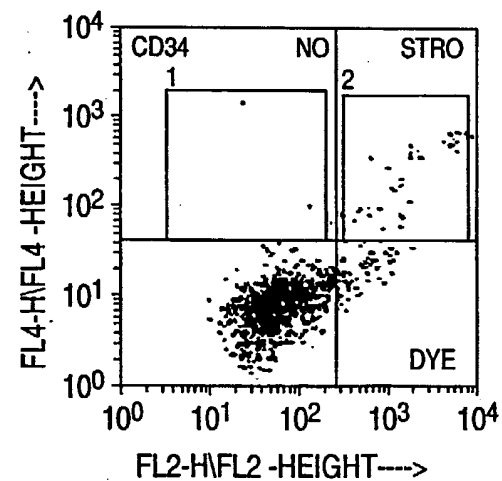


FIG. 8F



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FIG. 9A

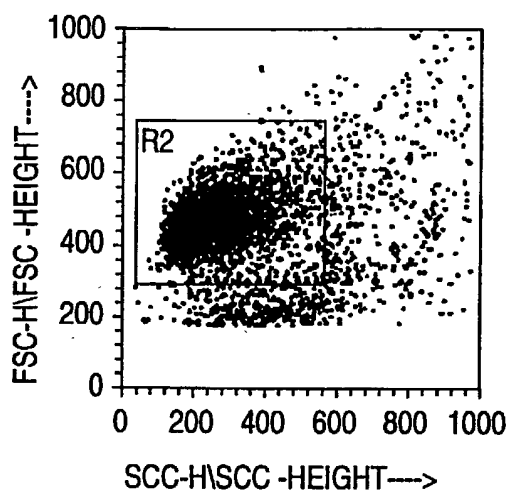


FIG. 9B

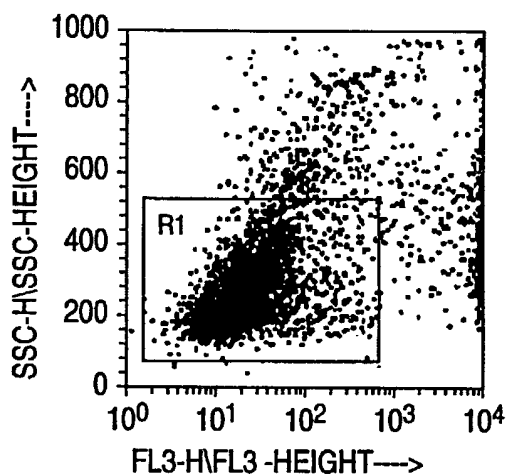
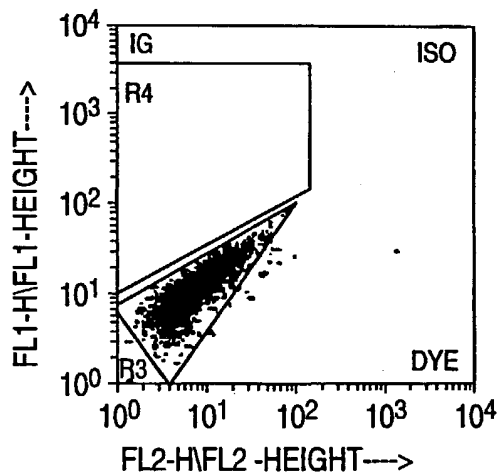


FIG. 9C



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FIG. 9D

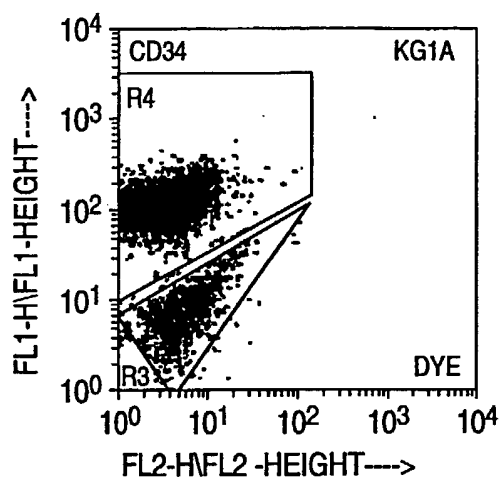


FIG. 9E

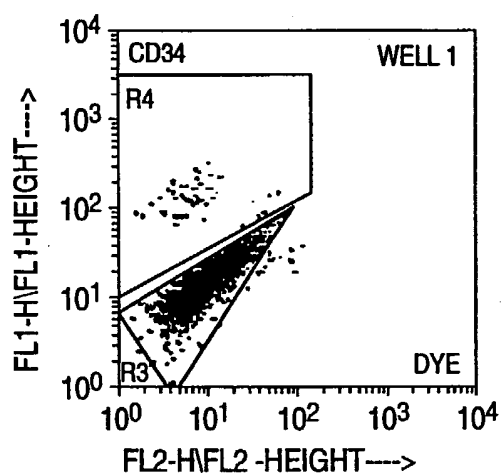
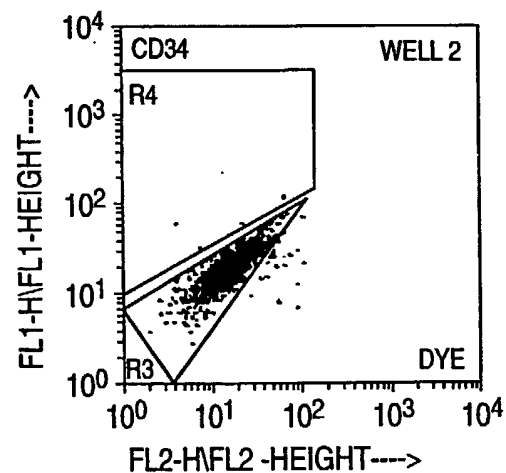


FIG. 9F



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FIG. 10A

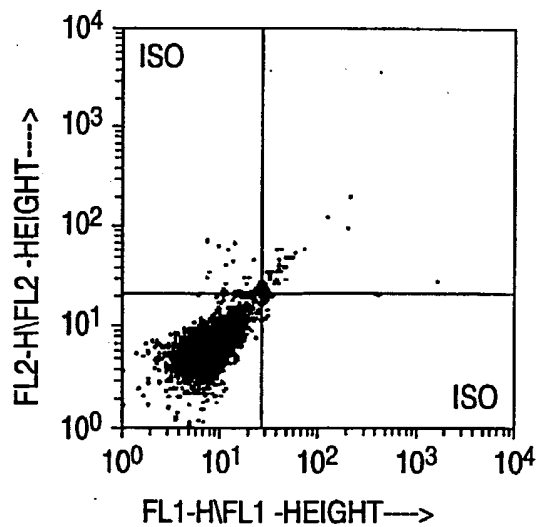
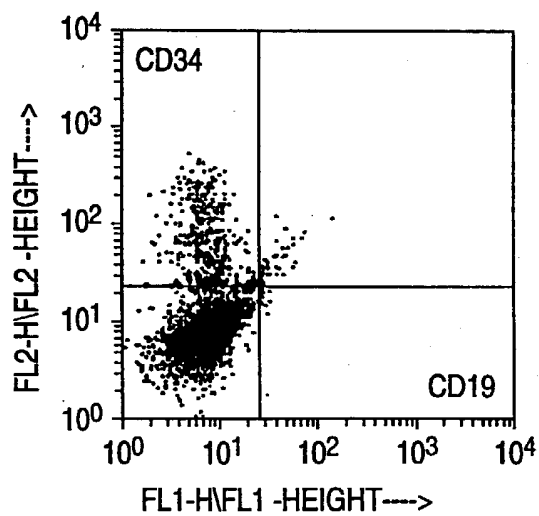


FIG. 10B



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AFTER BULK CLTR.ON STROMA
WELLS WITH GROWTH %

AFTER BULK CLTR NO STROMA
WELLS WITH GROWTH %

#7090 (FROZEN, CADAVERIC BM)

BRIGHT	77/144	53%	53/72	74%
DIM	39/144	27%	33/72	46%

#8653 (FRESH, ABM)

BRIGHT	52/72	72%	41/72	57%
DIM	14/72	19%	8/72	10%

#8415 (FRESH, MPB)

BRIGHT	38/63	60%
DIM	30/132	23%

#8814 (FRESH, MPB)

BRIGHT	37/72	51%	45/72	62%
DIM	26/72	36%	11/72	15%

FIG. 11

SUBSTITUTE SHEET (RULE 26)

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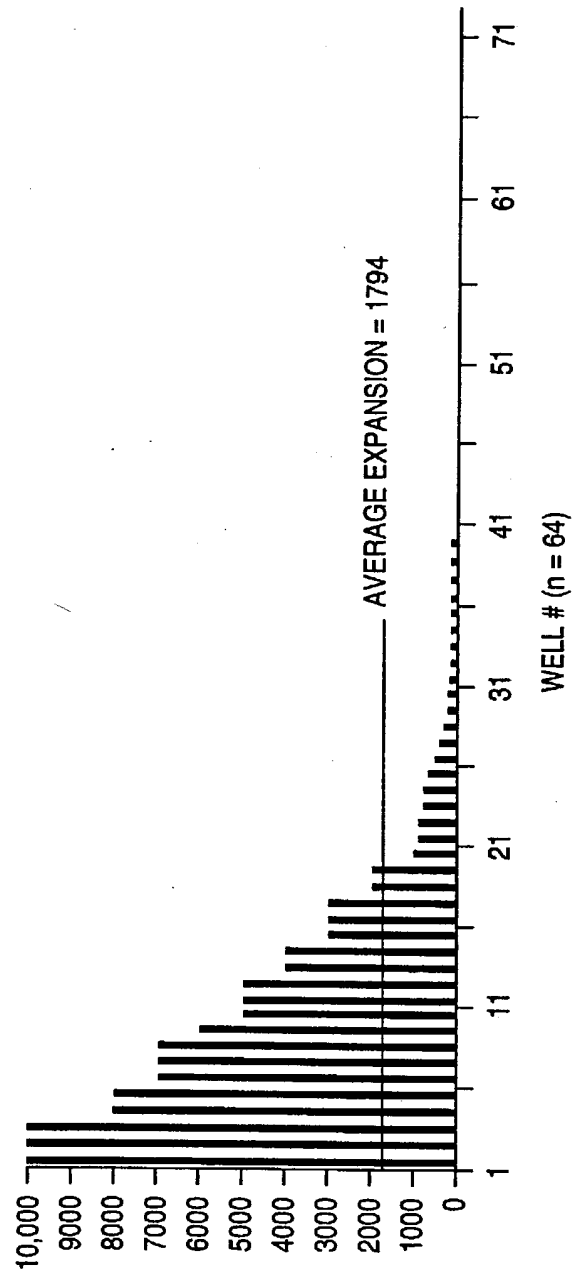


FIG. 12A

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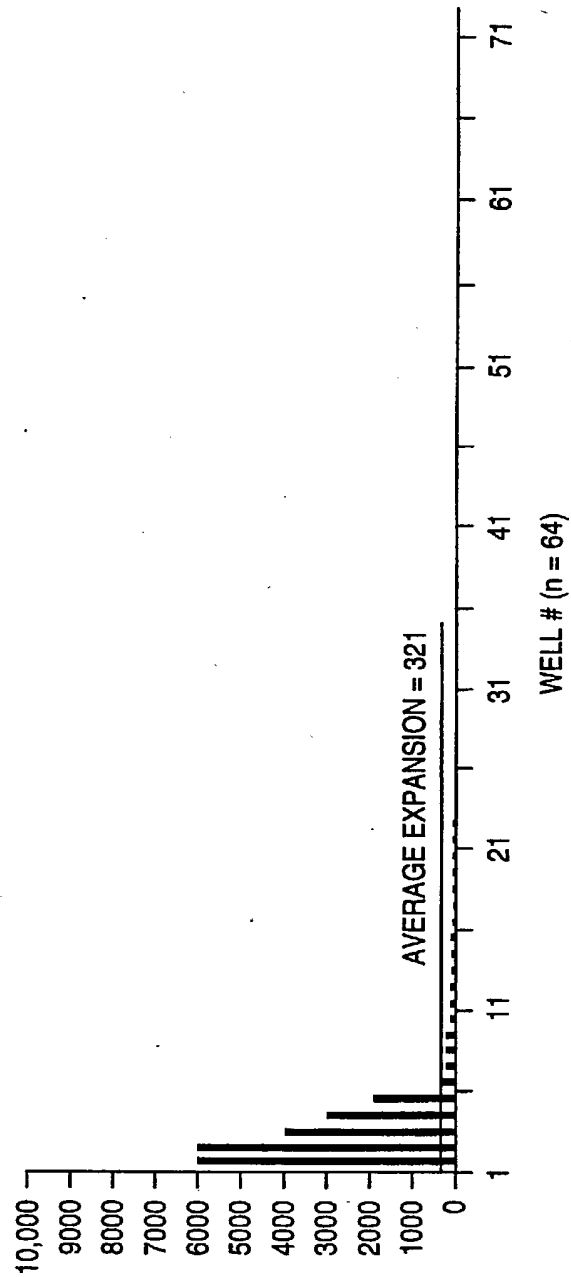


FIG. 12B

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<u>WELL #</u>	<u>AFTER BULK CLTR WITH STROMA</u>	<u>WELL #</u>	<u>AFTER BULK CLTR WITHOUT STROMA</u>
1	52 CFU-GM	8	18 CFU-GM
2	21 CFU-GM, 12 CFU-M, 2 CFU-MIX	9	0
3	58 CFU-GM, 10 CFU-M, 5 CFU-MIX	10	15 CFU-M
4	0	11	2 CFU-GM, 8 CFU-M
5	0	12	23 CFU-M
6	0		
7	6 CFU-GM		

FIG. 13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/11456

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 35/14; C12N 5/06, 5/08; G01N 33/533, 33/536

US CL : 424/93.7; 435/2, 7.24, 240.2, 240.23; 436/172, 174, 536, 546

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.7; 435/2, 7.24, 240.2, 240.23; 436/172, 174, 536, 546

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE

search terms: hematopoietic, stem cell, progenitor, FACS, stromal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proc. Natl. Acad. Sci. USA, Volume 89, issued April 1992, Baum et al, "Isolation of a candidate human hematopoietic stem-cell population", pages 2804-2808.	1-38, 40
Y	US, A, 5,199,942 (GILLIS) 06 April 1993, see entire document.	7, 8, 15-38, 40

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 21 DECEMBER 1994	Date of mailing of the international search report JAN 25 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY J. PARSONS <i>N. Parsons for</i> Telephone No. (703) 308-0196